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UTILITY PATENT
APPLICATION TRANSMITTAL LETTER



Box PATENT APPLICATION
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Enclosed for filing is the utility patent application of James ROBL, Jose CIBELLI, Steven L. STICE for EMBRYONIC OR STEM-LIKE CELL LINES PRODUCED BY CROSS SPECIES NUCLEAR TRANSPLANTATION AND METHODS FOR ENHANCING EMBRYONIC DEVELOPMENT BY GENETIC ALTERATION OF DONOR CELLS OR BY TISSUE CULTURE CONDITIONS.

Also enclosed are:

- ☒ Three sheet(s) of ☐ formal ☒ informal drawing(s);
- ☐ a claim for foreign priority under 35 U.S.C. §§ 119 and/or 365 is ☐ hereby made to ☐ filed in ☐ on ☐; ☐ in the declaration;
- ☐ a certified copy of the priority document;
- ☐ a Constructive Petition for Extensions of Time;
- ☐ ☐ statement(s) claiming small entity status;
- ☐ an Assignment document;
- ☐ an Information Disclosure Statement; and
- ☐ Other: ☐

The declaration of the inventor(s) ☐ also is enclosed ☒ will follow.

- ☐ Please amend the specification by inserting before the first line the sentence --This application claims priority under 35 U.S.C. §§119 and/or 365 to ☐ filed in ☐ on ☐; the entire content of which is hereby incorporated by reference.--

The filing fee has been calculated as follows [] and in accordance with the enclosed preliminary amendment:

CLAIMS					
	NO. OF CLAIMS		EXTRA CLAIMS	RATE	FEE
Basic Application Fee					\$760.00
Total Claims	50	MINUS 20 =	30	x \$18.00	540.00
Independent Claims	2	MINUS 3 =	0	x \$78.00	0.00
If multiple dependent claims are presented, add \$260.00					0.00
Total Application Fee					1300.00
If verified Statement claiming small entity status is enclosed, subtract 50% of Total Application Fee					650.00
Add Assignment Recording Fee of \$40.00 if Assignment document is enclosed					0.00
TOTAL APPLICATION FEE DUE					650.00

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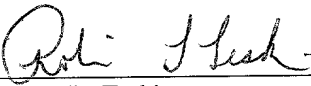
Robin L. Teskin
Burns, Doane, Swecker & Mathis, L.L.P.
P.O. Box 1404
Alexandria, Virginia 22313-1404.

The Commissioner is hereby authorized to charge any appropriate fees under 37 C.F.R. §§ 1.16, 1.17 and 1.21 that may be required by this paper, and to credit any overpayment, to Deposit Account No. 02-4800. This paper is submitted in triplicate.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

Date: March 2, 1999

By: 
Robin L. Teskin
Registration No. 35,030

P.O. Box 1404
Alexandria, Virginia 22313-1404
(703) 836-6620

EMBRYONIC OR STEM-LIKE CELL LINES PRODUCED BY
CROSS SPECIES NUCLEAR TRANSPLANTATION AND
METHODS FOR ENHANCING EMBRYONIC DEVELOPMENT
BY GENETIC ALTERATION OF DONOR CELLS OR
BY TISSUE CULTURE CONDITIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of application Serial No. 09/030,945, filed March 2, 1998, which in turn is a continuation-in-part of application Serial No. 08/699,040, filed August 19, 1996. This application is incorporated by reference in its entirety herein.

FIELD OF THE INVENTION

The present invention generally relates to the production of embryonic or stem-like cells by transplantation of cell nuclei derived from animal or human cells into enucleated animal oocytes of a species different from the donor nuclei. The present invention more specifically relates to the production of primate or human embryonic or stem-like cells by transplantation of the nucleus of a primate or human cell into an enucleated animal oocyte, e.g., a primate or ungulate oocyte and in a preferred embodiment a bovine enucleated oocyte.

The present invention further relates to the use of the resultant embryonic or stem-like cells, preferably primate or human embryonic or stem-like cells for therapy, for diagnostic applications, for the production of differentiated cells which may also be used for therapy or diagnosis, and for the production of transgenic embryonic or transgenic differentiated cells, cell lines, tissues and organs. Also, the embryonic or stem-like cells obtained according to the present invention may themselves be used as nuclear donors in nuclear transplantation

1 or nuclear transfer methods for the production of chimeras or clons, preferably
2 transgenic cloned or chimeric animals.

3 **BACKGROUND OF THE INVENTION**

4 Methods for deriving embryonic stem (ES) cell lines *in vitro* from early
5 preimplantation mouse embryos are well known. (See, e.g., Evans et al., *Nature*,
6 29:154-156 (1981); Martin, *Proc. Natl. Acad. Sci., USA*, 78:7634-7638 (1981)).
7 ES cells can be passaged in an undifferentiated state, provided that a feeder layer
8 of fibroblast cells (Evans et al., *Id.*) or a differentiation inhibiting source (Smith
9 et al., *Dev. Biol.*, 121:1-9 (1987)) is present.

10 ES cells have been previously reported to possess numerous applications.
11 For example, it has been reported that ES cells can be used as an *in vitro* model
12 for differentiation, especially for the study of genes which are involved in the
13 regulation of early development. Mouse ES cells can give rise to germline
14 chimeras when introduced into preimplantation mouse embryos, thus demon-
15 strating their pluripotency (Bradley et al., *Nature*, 309:255-256 (1984)).

16 In view of their ability to transfer their genome to the next generation, ES
17 cells have potential utility for germline manipulation of livestock animals by
18 using ES cells with or without a desired genetic modification. Moreover, in the
19 case of livestock animals, e.g., ungulates, nuclei from like preimplantation
20 livestock embryos support the development of enucleated oocytes to term (Smith
21 et al., *Biol. Reprod.*, 40:1027-1035 (1989); and Keefer et al., *Biol. Reprod.*,
22 50:935-939 (1994)). This is in contrast to nuclei from mouse embryos which
23 beyond the eight-cell stage after transfer reportedly do not support the
24 development of enucleated oocytes (Cheong et al., *Biol. Reprod.*, 48:958 (1993)).
25 Therefore, ES cells from livestock animals are highly desirable because they may

1 provide a potential source of totipotent donor nuclei, genetically manipulated or
2 otherwise, for nuclear transfer procedures.

3 Some research groups have reported the isolation of purportedly
4 pluripotent embryonic cell lines. For example, Notarianni et al., *J. Reprod. Fert.*
5 *Suppl.*, 43:255-260 (1991), report the establishment of purportedly stable,
6 pluripotent cell lines from pig and sheep blastocysts which exhibit some
7 morphological and growth characteristics similar to that of cells in primary
8 cultures of inner cell masses isolated immunosurgically from sheep blastocysts.
9 (*Id.*) Also, Notarianni et al., *J. Reprod. Fert. Suppl.*, 41:51-56 (1990) discloses
10 maintenance and differentiation in culture of putative pluripotential embryonic
11 cell lines from pig blastocysts. Further, Gerfen et al., *Anim. Biotech*, 6(1):1-14
12 (1995) disclose the isolation of embryonic cell lines from porcine blastocysts.
13 These cells are stably maintained in mouse embryonic fibroblast feeder layers
14 without the use of conditioned medium. These cells reportedly differentiate into
15 several different cell types during culture (Gerfen et al., *Id.*).

16 Further, Saito et al., *Roux's Arch. Dev. Biol.*, 201:134-141 (1992) report
17 bovine embryonic stem cell-like cell lines cultured which survived passages for
18 three, but were lost after the fourth passage. Still further, Handyside et al.,
19 *Roux's Arch. Dev. Biol.*, 196:185-190 (1987) disclose culturing of immunosurgi-
20 cally isolated inner cell masses of sheep embryos under conditions which allow
21 for the isolation of mouse ES cell lines derived from mouse ICMs. Handyside
22 et al. (1987) (*Id.*), report that under such conditions, the sheep ICMs attach,
23 spread, and develop areas of both ES cell-like and endoderm-like cells, but that
24 after prolonged culture only endoderm-like cells are evident. (*Id.*)

25 Recently, Cherny et al., *Theriogenology*, 41:175 (1994) reported
26 purportedly pluripotent bovine primordial germ cell-derived cell lines maintained

1 in long-term culture. These cells, after approximately seven days in culture,
2 produced ES-like colonies which stain positive for alkaline phosphatase (AP),
3 exhibited the ability to form embryoid bodies, and spontaneously differentiated
4 into at least two different cell types. These cells also reportedly expressed
5 mRNA for the transcription factors OCT4, OCT6 and HES1, a pattern of
6 homeobox genes which is believed to be expressed by ES cells exclusively.

7 Also recently, Campbell et al., *Nature*, 380:64-68 (1996) reported the
8 production of live lambs following nuclear transfer of cultured embryonic disc
9 (ED) cells from day nine ovine embryos cultured under conditions which
10 promote the isolation of ES cell lines in the mouse. The authors concluded based
11 on their results that ED cells from day nine ovine embryos are totipotent by
12 nuclear transfer and that totipotency is maintained in culture.

13 Van Stekelenburg-Hamers et al., *Mol. Reprod. Dev.*, 40:444-454 (1995),
14 reported the isolation and characterization of purportedly permanent cell lines
15 from inner cell mass cells of bovine blastocysts. The authors isolated and
16 cultured ICMs from 8 or 9 day bovine blastocysts under different conditions to
17 determine which feeder cells and culture media are most efficient in supporting
18 the attachment and outgrowth of bovine ICM cells. They concluded based on
19 their results that the attachment and outgrowth of cultured ICM cells is enhanced
20 by the use of STO (mouse fibroblast) feeder cells (instead of bovine uterus
21 epithelial cells) and by the use of charcoal-stripped serum (rather than normal se-
22 rum) to supplement the culture medium. Van Stekelenburg et al reported,
23 however, that their cell lines resembled epithelial cells more than pluripotent
24 ICM cells. (*Id.*)

25 Still further, Smith et al., WO 94/24274, published October 27, 1994,
26 Evans et al, WO 90/03432, published April 5, 1990, and Wheeler et al, WO

1 94/26889, published November 24, 1994, report the isolation, selection and
2 propagation of animal stem cells which purportedly may be used to obtain
3 transgenic animals. Also, Evans et al., WO 90/03432, published on April 5,
4 1990, reported the derivation of purportedly pluripotent embryonic stem cells
5 derived from porcine and bovine species which assertedly are useful for the
6 production of transgenic animals. Further, Wheeler et al, WO 94/26884,
7 published November 24, 1994, disclosed embryonic stem cells which are
8 assertedly useful for the manufacture of chimeric and transgenic ungulates.
9 Thus, based on the foregoing, it is evident that many groups have attempted to
10 produce ES cell lines, e.g., because of their potential application in the
11 production of cloned or transgenic embryos and in nuclear transplantation.

12 The use of ungulate ICM cells for nuclear transplantation has also been
13 reported. For example, Collas et al., *Mol. Reprod. Dev.*, 38:264-267 (1994)
14 disclose nuclear transplantation of bovine ICMs by microinjection of the lysed
15 donor cells into enucleated mature oocytes. The reference disclosed culturing of
16 embryos *in vitro* for seven days to produce fifteen blastocysts which, upon
17 transferral into bovine recipients, resulted in four pregnancies and two births.
18 Also, Keefer et al., *Biol. Reprod.*, 50:935-939 (1994), disclose the use of bovine
19 ICM cells as donor nuclei in nuclear transfer procedures, to produce blastocysts
20 which, upon transplantation into bovine recipients, resulted in several live
21 offspring. Further, Sims et al., *Proc. Natl. Acad. Sci., USA*, 90:6143-6147
22 (1993), disclosed the production of calves by transfer of nuclei from short-term
23 *in vitro* cultured bovine ICM cells into enucleated mature oocytes.

24 Also, the production of live lambs following nuclear transfer of cultured
25 embryonic disc cells has been reported (Campbell et al., *Nature*, 380:64-68
26 (1996)). Still further, the use of bovine pluripotent embryonic cells in nuclear

1 transfer and the production of chimeric fetuses has also been reported (Stice
2 et al., *Biol. Reprod.*, 54:100-110 (1996)); Collas et al, *Mol. Reprod. Dev.*,
3 38:264-267 (1994).

4 Also, there have been previous attempts to produce cross species NT units
5 (Wolfe et al., *Theriogenology*, 33:350 (1990). Specifically, bovine embryonic
6 cells were fused with bison oocytes to produce some cross species NT units
7 possibly having an inner cell mass. However, embryonic cells, not adult cells
8 were used, as donor nuclei in the nuclear transfer procedure. The dogma has
9 been that embryonic cells are more easily reprogrammed than adult cells. This
10 dates back to earlier NT studies in the frog (review by DiBerardino,
11 *Differentiation*, 17:17-30 (1980)). Also, this study involved very
12 phylogenetically similar animals (cattle nuclei and bison oocytes). By contrast,
13 previously when more diverse species were fused during NT (cattle nuclei into
14 hamster oocytes), no inner cell mass structures were obtained. Further, it has
15 never been previously reported that the inner cell mass cells from NT units could
16 be used to form an ES cell-like colony that could be propagated.

17 Also, Collas et al (*Id.*), taught the use of granulosa cells (adult somatic
18 cells) to produce bovine nuclear transfer embryos. However, unlike the present
19 invention, these experiments did not involve cross-species nuclear transfer.
20 Also, unlike the present invention ES-like cell colonies were not obtained.

21 Very recently, U.S. Patent No. 5,843,780, issued to James A. Thomson
22 on December 1, 1998, assigned to the Wisconsin Alumni Research Foundation,
23 that purports to disclose a purified preparation of primate embryonic stem cells
24 that are (i) capable of proliferation in an *in vitro* culture for over one year; (ii)
25 maintain a karyotype in which all chromosomes characteristic of the primate
26 species are present and not noticeably altered through prolonged culture; (iii)

1 maintains the potential to differentiate into derivatives of endoderm, mesoderm
2 and ectoderm tissues throughout culture; and (iv) will not differentiate when
3 cultured on a fibroblast feeder layer. These cells were reportedly negative for the
4 SSEA-1 marker, positive for the SEA-3 marker, positive for the SSEA-4 marker,
5 express alkaline phosphatase activity, are pluripotent, and have karyotypes which
6 include the presence of all the chromosomes characteristic of the primate species
7 and in which none of the chromosomes are altered. Further, these cells are
8 respectfully positive for the TRA-1-60, and TRA-1-81 markers. The cells
9 purportedly differentiate into endoderm, mesoderm and ectoderm cells when
10 injected into a SCID mouse. Also, purported embryonic stem cell lines derived
11 from human or primate blastocytes are discussed in Thomson et al., Science
12 282:1145-1147 and Proc. Natl. Acad. Sci., USA 92:7844-7848 (1995).

13 Thus, Thomson disclose what purportedly are non-human primate and
14 human embryonic or stem-like cells and methods for their production. However,
15 there still exists a significant need for methods for producing human embryonic
16 or stem-like cells that are autologous to an intended transplant recipient given
17 their significant therapeutic and diagnostic potential.

18 In this regard, numerous human diseases have been identified which may
19 be treated by cell transplantation. For example, Parkinson's disease is caused by
20 degeneration of dopaminergic neurons in the substantia nigra. Standard
21 treatment for Parkinson's involves administration of L-DOPA, which temporarily
22 ameliorates the loss of dopamine, but causes severe side effects and ultimately
23 does not reverse the progress of the disease. A different approach to treating
24 Parkinson's, which promises to have broad applicability to treatment of many
25 brain diseases and central nervous system injury, involves transplantation of cells
26 or tissues from fetal or neonatal animals into the adult brain. Fetal neurons from

1 a variety of brain regions can be incorporated into the adult brain. Such grafts
2 have been shown to alleviate experimentally induced behavioral deficits, includ-
3 ing complex cognitive functions, in laboratory animals. Initial test results from
4 human clinical trials have also been promising. However, supplies of human
5 fetal cells or tissue obtained from miscarriages is very limited. Moreover,
6 obtaining cells or tissues from aborted fetuses is highly controversial.

7 There is currently no available procedure for producing "fetal-like" cells
8 from the patient. Further, allograft tissue is not readily available and both allo-
9 graft and xenograft tissue are subject to graft rejection. Moreover, in some cases,
10 it would be beneficial to make genetic modifications in cells or tissues before
11 transplantation. However, many cells or tissues wherein such modification
12 would be desirable do not divide well in culture and most types of genetic
13 transformation require rapidly dividing cells.

14 There is therefore a clear need in the art for a supply of human embryonic
15 or stem-like undifferentiated cells for use in transplants and cell and gene thera-
16 pies.

17 **OBJECTS OF THE INVENTION**

18 It is an object of the invention to provide novel and improved methods for
19 producing embryonic or stem-like cells.

20 It is a more specific object of the invention to provide a novel method for
21 producing embryonic or stem-like cells which involves transplantation of the
22 nucleus of a mammalian or human cell into an enucleated oocyte of a different
23 species.

24 It is another specific object of the invention to provide a novel method for
25 producing non-human primate or human embryonic or stem-like cells which
26 involves transplantation of the nucleus of a non-human primate or human cell

1 into an enucleated animal or human oocyte, e.g., an ungulate, human or primate
2 enucleated oocyte.

3 It is another object of the invention to provide a novel method for
4 producing lineage-defective non-human primate or human embryonic or stem-
5 like cells which involves transplantation of the nucleus of a non-human primate
6 or human cell, e.g., a human adult cell into an enucleated non-human primate or
7 human oocyte, wherein such cell has been genetically engineered to be incapable
8 of differentiation into a specific cell lineage or has been modified such that the
9 cells are "mortal", and thereby do not give rise to a viable offspring, e.g., by
10 engineering expression of anti-sense or ribozyme telomerase gene.

11 It is still another object of the invention to enhance efficiency of nuclear
12 transfer and specifically to enhance the development of preimplantation embryos
13 produced by nuclear transfer by genetically engineering donor somatic cells used
14 for nuclear transfer to provide for the expression of genes that enhance
15 embryonic development, e.g., genes of the MHC I family, and in particular Ped
16 genes such as Q7 and/or Q9.

17 It is yet another object of the invention to enhance the production of
18 nuclear transfer embryos by IVP and more specifically nuclear transfer embryos
19 by genetically altering the donor cell used for nuclear transfer such that it is
20 resistant to apoptosis, e.g., by introduction of a DNA construct that provides for
21 the expression of genes that inhibit apoptosis, e.g., Bcl-2 or Bcl-2 family
22 members and/or by the expression of antisense ribozymes specific to genes that
23 induce apoptosis during early embryonic development.

24 It is still another object of the invention to improve the efficacy of nuclear
25 transfer by improved selection of donor cells of a specific cell cycle stage, e.g.,
26 G1 phase, by genetically engineering donor cells such that they express a DNA

1 construct encoding a particular cyclin linked to a detectable marker, e.g., one that
2 encodes a visualizable (e.g., fluorescent tag) marker protein.

3 It is also an object of the invention to enhance the development of *in vitro*
4 produced embryos, by culturing such embryos in the presence of one or more
5 protease inhibitors, preferably one or more caspase inhibitors, thereby inhibiting
6 apoptosis.

7 It is another object of the invention to provide embryonic or stem-like
8 cells produced by transplantation of nucleus of an animal or human cell into an
9 enucleated oocyte of a different species.

10 It is a more specific object of the invention to provide primate or human
11 embryonic or stem-like cells produced by transplantation of the nucleus of a
12 primate or human cell into an enucleated animal oocyte, e.g., a human, primate
13 or ungulate enucleated oocyte.

14 It is another object of the invention to use such embryonic or stem-like
15 cells for therapy or diagnosis.

16 It is a specific object of the invention to use such primate or human
17 embryonic or stem-like cells for treatment or diagnosis of any disease wherein
18 cell, tissue or organ transplantation is therapeutically or diagnostically beneficial.

19 It is another specific object of the invention to use the embryonic or stem-
20 like cells produced according to the invention for the production of differentiated
21 cells, tissues or organs.

22 It is a more specific object of the invention to use the primate or human
23 embryonic or stem-like cells produced according to the invention for the
24 production of differentiated human cells, tissues or organs.

25 It is another specific object of the invention to use the embryonic or stem-
26 like cells produced according to the invention for the production of genetically

1 engineered embryonic or stem-like cells, which cells may be used to produce
2 genetically engineered or transgenic differentiated human cells, tissues or organs,
3 e.g., having use in gene therapies.

4 It is another specific object of the invention to use the embryonic or stem-
5 like cells produced according to the invention *in vitro*, e.g. for study of cell dif-
6 ferentiation and for assay purposes, e.g. for drug studies.

7 It is another object of the invention to provide improved methods of
8 transplantation therapy, comprising the usage of isogenic or syngenic cells,
9 tissues or organs produced from the embryonic or stem-like cells produced
10 according to the invention. Such therapies include by way of example treatment
11 of diseases and injuries including Parkinson's, Huntington's, Alzheimer's, ALS,
12 spinal cord injuries, multiple sclerosis, muscular dystrophy, diabetes, liver
13 diseases, heart disease, cartilage replacement, burns, vascular diseases, urinary
14 tract diseases, as well as for the treatment of immune defects, bone marrow trans-
15 plantation, cancer, among other diseases.

16 It is another object of the invention to use the transgenic or genetically
17 engineered embryonic or stem-like cells produced according to the invention for
18 gene therapy, in particular for the treatment and/or prevention of the diseases and
19 injuries identified, *supra*.

20 It is another object of the invention to use the embryonic or stem-like cells
21 produced according to the invention or transgenic or genetically engineered
22 embryonic or stem-like cells produced according to the invention as nuclear
23 donors for nuclear transplantation.

24 It is still another object of the invention to use genetically engineered ES
25 cells produced according to the invention for the production of transgenic
26 animals, e.g., non-human primates, rodents, ungulates, etc. Such transgenic

1 animals can be used to produce, e.g., animal models for study of human diseases,
2 or for the production of desired polypeptides, e.g., therapeutics or
3 nutraceuticals.

4 With the foregoing and other objects, advantages and features of the
5 invention that will become hereinafter apparent, the nature of the invention may
6 be more clearly understood by reference to the following detailed description of
7 the preferred embodiments of the invention and to the appended claims.

8 **BRIEF DESCRIPTION OF THE FIGURES**

9 Figure 1 is a photograph of a nuclear transfer (NT) unit produced by
10 transfer of an adult human cell into an enucleated bovine oocyte.

11 Figures 2 to 5 are photographs of embryonic stem-like cells derived from
12 a NT unit such as is depicted in Figure 1.

13 **DETAILED DESCRIPTION OF THE INVENTION**

14 The present invention provides a novel method for producing embryonic
15 or stem-like cells, and more specifically non-human primate or human
16 embryonic or stem-like cells by nuclear transfer or nuclear transplantation. In
17 the subject application, nuclear transfer or nuclear transplantation or NT are used
18 interchangeably.

19 As discussed *supra*, the isolation of actual embryonic or stem-like cells
20 by nuclear transfer or nuclear transplantation has never been reported. Rather,
21 previous reported isolation of ES-like cells has been from fertilized embryos.
22 Also, successful nuclear transfer involving cells or DNA of genetically dissimilar
23 species, or more specifically adult cells or DNA of one species (e.g., human) and
24 oocytes of another non-related species has never been reported. Rather, while
25 embryos produced by fusion of cells of closely related species, has been reported,
26 e.g., bovine-goat and bovine-bison, they did not produce ES cells. (Wolfe et al,

1 Theriogenology, 33(1):350 (1990).) Also, there has never been reported a
2 method for producing primate or human ES cells derived from a non-fetal tissue
3 source. Rather, the limited human fetal cells and tissues which are currently
4 available must be obtained or derived from spontaneous abortion tissues and
5 from aborted fetuses.

6 Also, prior to the present invention, no one obtained embryonic or stem-
7 like cells by cross-species nuclear transplantation.

8 Quite unexpectedly, the present inventors discovered that human
9 embryonic or stem-like cells and cell colonies may be obtained by
10 transplantation of the nucleus of a human cell, e.g., an adult differentiated human
11 cell, into an enucleated animal oocyte, which is used to produce nuclear transfer
12 (NT) units, the cells of which upon culturing give rise to human embryonic or
13 stem-like cells and cell colonies. This result is highly surprising because it is the
14 first demonstration of effective cross-species nuclear transplantation involving
15 the introduction of a differentiated donor cell or nucleus into an enucleated
16 oocyte of a genetically dissimilar species, e.g., the transplantation of cell nuclei
17 from a differentiated animal or human cell, e.g., adult cell, into the enucleated
18 egg of a different animal species, to produce nuclear transfer units containing
19 cells which when cultured under appropriate conditions give rise to embryonic
20 or stem-like cells and cell colonies.

21 Preferably, the NT units used to produce ES-like cells will be cultured to
22 a size of at least 2 to 400 cells, preferably 4 to 128 cells, and most preferably to
23 a size of at least about 50 cells.

24 In the present invention, embryonic or stem-like cells refer to cells
25 produced according to the present invention. The present application refers to
26 such cells as stem-like cells rather than stem cells because of the manner in

1 which they are typically produced, i.e., by cross-species nuclear transfer. While
2 these cells are expected to possess similar differentiation capacity as normal stem
3 cells they may possess some insignificant differences because of the manner they
4 are produced. For example, these stem-like cells may possess the mitochondria
5 of the oocytes used for nuclear transfer, and thus not behave identically to
6 conventional embryonic stem cells.

7 The present discovery was made based on the observation that nuclear
8 transplantation of the nucleus of an adult human cell, specifically a human
9 epithelial cell obtained from the oral cavity of a human donor, when transferred
10 into an enucleated bovine oocyte, resulted in the formation of nuclear transfer
11 units, the cells of which upon culturing gave rise to human stem-like or embry-
12 onic cells and human embryonic or stem-like cell colonies. This result has
13 recently been reproduced by transplantation of keratinocytes from an adult
14 human into an enucleated bovine oocyte with the successful production of a
15 blastocyst and ES cell line. Based thereon, it is hypothesized by the present
16 inventors that bovine oocytes and human oocytes, and likely mammals in general
17 must undergo maturation processes during embryonic development which are
18 sufficiently similar or conserved so as to permit the bovine oocyte to function as
19 an effective substitute or surrogate for a human oocyte. Apparently, oocytes in
20 general comprise factors, likely proteinaceous or nucleic acid in nature, that
21 induce embryonic development under appropriate conditions, and these functions
22 that are the same or very similar in different species. These factors may
23 comprise material RNAs and/or telomerase.

24 Based on the fact that human cell nuclei can be effectively transplanted
25 into bovine oocytes, it is reasonable to expect that human cells may be
26 transplanted into oocytes of other non-related species, e.g., other ungulates as

1 well as other animals. In particular, other ungulate oocytes should be suitable,
2 e.g. pigs, sheep, horses, goats, etc. Also, oocytes from other sources should be
3 suitable, e.g. oocytes derived from other primates, amphibians, rodents, rabbits,
4 guinea pigs, etc. Further, using similar methods, it should be possible to transfer
5 human cells or cell nuclei into human oocytes and use the resultant blastocytes
6 to produce human ES cells.

7 Therefore, in its broadest embodiment, the present invention involves the
8 transplantation of an animal or human cell nucleus or animal or human cell into
9 the enucleated oocyte of an animal species different from the donor nuclei, by
10 injection or fusion, to produce an NT unit containing cells which may be used to
11 obtain embryonic or stem-like cells and/or cell cultures. For example, the
12 invention may involve the transplantation of an ungulate cell nucleus or ungulate
13 cell into an enucleated oocyte of another species, e.g., another ungulate or non-
14 ungulate, by injection or fusion, which cells and/or nuclei are combined to
15 produce NT units and which are cultured under conditions suitable to obtain
16 multicellular NT units, preferably comprising at least about 2 to 400 cells, more
17 preferably 4 to 128 cells, and most preferably at least about 50 cells. The cells
18 of such NT units may be used to produce embryonic or stem-like cells or cell
19 colonies upon culturing.

20 However, the preferred embodiment of the invention comprises the
21 production of non-human primate or human embryonic or stem-like cells by
22 transplantation of the nucleus of a donor human cell or a human cell into an
23 enucleated human, primate, or non-primate animal oocyte, e.g., an ungulate
24 oocyte, and in a preferred embodiment a bovine enucleated oocyte.

25 In general, the embryonic or stem-like cells will be produced by a nuclear
26 transfer process comprising the following steps:

1 (i) obtaining desired human or animal cells to be used as a source of
2 donor nuclei (which may be genetically altered);

3 (ii) obtaining oocytes from a suitable source, e.g. a mammal and most
4 preferably a primate or an ungulate source, e.g. bovine,

5 (iii) enucleating said oocytes;

6 (iv) transferring the human or animal cell or nucleus into the enucleated
7 oocyte of an animal species different than the donor cell or nuclei, e.g., by fusion
8 or injection;

9 (v) culturing the resultant NT product or NT unit to produce multiple cell
10 structures; and

11 (vi) culturing cells obtained from said embryos to obtain embryonic or
12 stem-like cells and stem-like cell colonies.

13 Nuclear transfer techniques or nuclear transplantation techniques are
14 known in the literature and are described in many of the references cited in the
15 Background of the Invention. See, in particular, Campbell et al, *Theriogenology*,
16 43:181 (1995); Collas et al, *Mol. Report Dev.*, 38:264-267 (1994); Keefer et al,
17 *Biol. Reprod.*, 50:935-939 (1994); Sims et al, *Proc. Natl. Acad. Sci., USA*,
18 90:6143-6147 (1993); WO 94/26884; WO 94/24274, and WO 90/03432, which
19 are incorporated by reference in their entirety herein. Also, U.S. Patent Nos.
20 4,944,384 and 5,057,420 describe procedures for bovine nuclear transplantation.
21 See, also Cibelli et al, *Science*, Vol. 280:1256-1258 (1998).

22 Human or animal cells, preferably mammalian cells, may be obtained and
23 cultured by well known methods. Human and animal cells useful in the present
24 invention include, by way of example, epithelial, neural cells, epidermal cells,
25 keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes (B
26 and T lymphocytes), other immune cells, erythrocytes, macrophages,

1 melanocytes, monocytes, mononuclear cells, fibroblasts, cardiac muscle cells,
2 and other muscle cells, etc. Moreover, the human cells used for nuclear transfer
3 may be obtained from different organs, e.g., skin, lung, pancreas, liver, stomach,
4 intestine, heart, reproductive organs, bladder, kidney, urethra and other urinary
5 organs, etc. These are just examples of suitable donor cells. Suitable donor
6 cells, i.e., cells useful in the subject invention, may be obtained from any cell or
7 organ of the body. This includes all somatic or germ cells. Preferably, the donor
8 cells or nucleus would comprise actively dividing, i.e., non-quiescent, cells as
9 this has been reported to enhance cloning efficacy. Also preferably, such donor
10 cells will be in the G1 cell cycle.

11 The resultant blastocytes may be used to obtain embryonic stem cell lines
12 according to the culturing methods reported by Thomson et al., Science
13 282:1145-1147 (1998) and Thomson et al., Proc. Natl. Acad. Sci., USA 92:7544-
14 7848 (1995), incorporated by reference in their entirety herein.

15 In the example which follows the cells used as donors for nuclear transfer
16 were epithelial cells derived from the oral cavity of a human donor and adult
17 human keratinocytes. However, as discussed, the disclosed method is applicable
18 to other human cells or nuclei. Moreover, the cell nuclei may be obtained from
19 both human somatic and germ cells.

20 It is also possible to arrest donor cells at mitosis before nuclear transfer,
21 using a suitable technique known in the art. Methods for stopping the cell cycle
22 at various stages have been thoroughly reviewed in U.S. Patent 5,262,409, which
23 is herein incorporated by reference. In particular, while cycloheximide has been
24 reported to have an inhibitory effect on mitosis (Bowen and Wilson (1955) J.
25 Heredity 45: 3-9), it may also be employed for improved activation of mature

1 bovine follicular oocytes when combined with electric pulse treatment (Yang et
2 al. (1992) Biol. Reprod. 42 (Suppl. 1): 117).

3 Zygote gene activation is associated with hyperacetylation of Histone H4.
4 Trichostatin-A has been shown to inhibit histone deacetylase in a reversible
5 manner (Adenot et al. Differential H4 acetylation of paternal and maternal
6 chromatin precedes DNA replication and differential transcriptional activity in
7 pronuclei of 1-cell mouse embryos. Development (Nov. 1997) 124(22): 4615-
8 4625; Yoshida et al. Trichostatin A and trapoxin: novel chemical probes for the
9 role of histone acetylation in chromatin structure and function. Bioessays (May,
10 1995) 17(5): 423-430), as have other compounds.

11 For instance, butyrate is also believed to cause hyper-acetylations of
12 histones by inhibiting histone deacetylase. Generally, butyrate appears to modify
13 gene expression and in almost all cases its addition to cells in culture appears to
14 arrest cell growth. Use of butyrate in this regard is described in U.S. Patent No.
15 5,681,718, which is herein incorporated by reference. Thus, donor cells may be
16 exposed to Trichostatin-A or another appropriate deacetylase inhibitor prior to
17 fusion, or such a compound may be added to the culture media prior to genome
18 activation.

19 Additionally, demethylation of DNA is thought to be a requirement for
20 proper access of transcription factors to DNA regulatory sequences. Global
21 demethylation of DNA from the eight-cell stage to the blastocyst stage in
22 preimplantation embryos has previously been described (Stein et al., Mol.
23 Reprod. & Dev. 47(4): 421-429). Also, Jaenisch et al. (1997) have reported that
24 5-azacytidine can be used to reduce the level of DNA methylation in cells,
25 potentially leading to increased access of transcription factors to DNA regulatory
26 sequences. Accordingly, donor cells may be exposed to 5-azacytidine (5-Aza)

1 previous to fusion, or 5-Aza may be added to the culture medium from the 8 cell
2 stage to blastocyst. Alternatively, other known methods for effecting DNA
3 demethylation may be used.

4 The oocytes used for nuclear transfer may be obtained from animals
5 including mammals and amphibians. Suitable mammalian sources for oocytes
6 include sheep, bovines, ovines, pigs, horses, rabbits, goats, guinea pigs, mice,
7 hamsters, rats, primates, humans, etc. In the preferred embodiments, the oocytes
8 will be obtained from primates or ungulates, e.g., a bovine.

9 Methods for isolation of oocytes are well known in the art. Essentially,
10 this will comprise isolating oocytes from the ovaries or reproductive tract of a
11 mammal or amphibian, e.g., a bovine. A readily available source of bovine
12 oocytes is slaughterhouse materials.

13 For the successful use of techniques such as genetic engineering, nuclear
14 transfer and cloning, oocytes must generally be matured *in vitro* before these
15 cells may be used as recipient cells for nuclear transfer, and before they can be
16 fertilized by the sperm cell to develop into an embryo. This process generally
17 requires collecting immature (prophase I) oocytes from animal ovaries, e.g.,
18 bovine ovaries obtained at a slaughterhouse and maturing the oocytes in a
19 maturation medium prior to fertilization or enucleation until the oocyte attains
20 the metaphase II stage, which in the case of bovine oocytes generally occurs
21 about 18-24 hours post-aspiration. For purposes of the present invention, this
22 period of time is known as the "maturation period." As used herein for
23 calculation of time periods, "aspiration" refers to aspiration of the immature
24 oocyte from ovarian follicles.

25 Additionally, metaphase II stage oocytes, which have been matured *in*
26 *vivo* have been successfully used in nuclear transfer techniques. Essentially,

1 mature metaphase II oocytes are collected surgically from either non-superovu-
2 lated or superovulated cows or heifers 35 to 48 hours past the onset of estrus or
3 past the injection of human chorionic gonadotropin (hCG) or similar hormone.

4 The stage of maturation of the oocyte at enucleation and nuclear transfer
5 has been reported to be significant to the success of NT methods. (See e.g.,
6 Prather et al., *Differentiation*, 48, 1-8, 1991). In general, previous successful
7 mammalian embryo cloning practices use the metaphase II stage oocyte as the
8 recipient oocyte because at this stage it is believed that the oocyte can be or is
9 sufficiently "activated" to treat the introduced nucleus as it does a fertilizing
10 sperm. In domestic animals, and especially cattle, the oocyte activation period
11 generally ranges from about 16-52 hours, preferably about 28-42 hours post-
12 aspiration.

13 For example, immature oocytes may be washed in HEPES buffered
14 hamster embryo culture medium (HECM) as described in Seshagine et al., *Biol.*
15 *Reprod.*, 40, 544-606, 1989, and then placed into drops of maturation medium
16 consisting of 50 microliters of tissue culture medium (TCM) 199 containing 10%
17 fetal calf serum which contains appropriate gonadotropins such as luteinizing
18 hormone (LH) and follicle stimulating hormone (FSH), and estradiol under a
19 layer of lightweight paraffin or silicon at 39°C.

20 After a fixed time maturation period, which typically will range from
21 about 10 to 40 hours, and preferably about 16-18 hours, the oocytes will be
22 enucleated. Prior to enucleation the oocytes will preferably be removed and
23 placed in HECM containing 1 milligram per milliliter of hyaluronidase prior to
24 removal of cumulus cells. This may be effected by repeated pipetting through
25 very fine bore pipettes or by vortexing briefly. The stripped oocytes are then
26 screened for polar bodies, and the selected metaphase II oocytes, as determined

1 by the presence of polar bodies, are then used for nuclear transfer. Enucleation
2 follows.

3 Enucleation may be effected by known methods, such as described in U.S.
4 Patent No. 4,994,384 which is incorporated by reference herein. For example,
5 metaphase II oocytes are either placed in HECM, optionally containing 7.5
6 micrograms per milliliter cytochalasin B, for immediate enucleation, or may be
7 placed in a suitable medium, for example CR1aa, plus 10% estrus cow serum,
8 and then enucleated later, preferably not more than 24 hours later, and more
9 preferably 16-18 hours later.

10 Enucleation may be accomplished microsurgically using a micropipette
11 to remove the polar body and the adjacent cytoplasm. The oocytes may then be
12 screened to identify those of which have been successfully enucleated. This
13 screening may be effected by staining the oocytes with 1 microgram per milliliter
14 33342 Hoechst dye in HECM, and then viewing the oocytes under ultraviolet
15 irradiation for less than 10 seconds. The oocytes that have been successfully
16 enucleated can then be placed in a suitable culture medium.

17 In the present invention, the recipient oocytes will preferably be
18 enucleated at a time ranging from about 10 hours to about 40 hours after the
19 initiation of *in vitro* maturation, more preferably from about 16 hours to about
20 24 hours after initiation of *in vitro* maturation, and most preferably about 16-18
21 hours after initiation of *in vitro* maturation.

22 A single animal or human cell or nucleus derived therefrom which is
23 typically heterologous to the enucleated oocyte will then be transferred into the
24 perivitelline space of the enucleated oocyte used to produce the NT unit. The
25 animal or human cell or nucleus and the enucleated oocyte will be used to
26 produce NT units according to methods known in the art. For example, the cells

1 may be fused by electrofusion. Electrofusion is accomplished by providing a
2 pulse of electricity that is sufficient to cause a transient break down of the plasma
3 membrane. This breakdown of the plasma membrane is very short because the
4 membrane reforms rapidly. Essentially, if two adjacent membranes are induced
5 to break down, upon reformation the lipid bilayers intermingle and small chan-
6 nels will open between the two cells. Due to the thermodynamic instability of
7 such a small opening, it enlarges until the two cells become one. Reference is
8 made to U.S. Patent 4,997,384 by Prather et al., (incorporated by reference in its
9 entirety herein) for a further discussion of this process. A variety of electro-
10 fusion media can be used including e.g., sucrose, mannitol, sorbitol and phos-
11 phate buffered solution. Fusion can also be accomplished using Sendai virus as
12 a fusogenic agent (Graham, *Wister Inot. Symp. Monogr.*, 9, 19, 1969).

13 Also, in some cases (e.g. with small donor nuclei) it may be preferable to
14 inject the nucleus directly into the oocyte rather than using electroporation
15 fusion. Such techniques are disclosed in Collas and Barnes, *Mol. Reprod. Dev.*,
16 38:264-267 (1994), and incorporated by reference in its entirety herein.

17 Preferably, the human or animal cell and oocyte are electrofused in a 500
18 μ m chamber by application of an electrical pulse of 90-120V for about 15 μ sec,
19 about 24 hours after initiation of oocyte maturation. After fusion, the resultant
20 fused NT units are then placed in a suitable medium until activation, e.g., one
21 identified *infra*. Typically activation will be effected shortly thereafter, typically
22 less than 24 hours later, and preferably about 4-9 hours later.

23 The NT unit may be activated by known methods. Such methods include,
24 e.g., culturing the NT unit at sub-physiological temperature, in essence by
25 applying a cold, or actually cool temperature shock to the NT unit. This may be
26 most conveniently done by culturing the NT unit at room temperature, which is

1 cold relative to the physiological temperature conditions to which embryos are
2 normally exposed.

3 Alternatively, activation may be achieved by application of known
4 activation agents. For example, penetration of oocytes by sperm during
5 fertilization has been shown to activate prefusion oocytes to yield greater
6 numbers of viable pregnancies and multiple genetically identical calves after
7 nuclear transfer. Also, treatments such as electrical and chemical shock or
8 cycloheximide treatment may also be used to activate NT embryos after fusion.
9 Suitable oocyte activation methods are the subject of U.S. Patent No. 5,496,720,
10 to Susko-Parrish et al., which is herein incorporated by reference.

11 For example, oocyte activation may be effected by simultaneously or
12 sequentially:

- 13 (i) increasing levels of divalent cations in the oocyte, and
14 (ii) reducing phosphorylation of cellular proteins in the oocyte.

15 This will generally be effected by introducing divalent cations into the
16 oocyte cytoplasm, e.g., magnesium, strontium, barium or calcium, e.g., in the
17 form of an ionophore. Other methods of increasing divalent cation levels include
18 the use of electric shock, treatment with ethanol and treatment with caged
19 chelators.

20 Phosphorylation may be reduced by known methods, e.g., by the addition
21 of kinase inhibitors, e.g., serine-threonine kinase inhibitors, such as 6-dimethyl-
22 amino-purine, staurosporine, 2-aminopurine, and sphingosine.

23 Alternatively, phosphorylation of cellular proteins may be inhibited by
24 introduction of a phosphatase into the oocyte, e.g., phosphatase 2A and
25 phosphatase 2B.

Activated NT units may be cultured in a suitable *in vitro* culture medium until the generation of embryonic or stem-like cells and cell colonies. Culture media suitable for culturing and maturation of embryos are well known in the art. Examples of known media, which may be used for bovine embryo culture and maintenance, include Ham's F-10 + 10% fetal calf serum (FCS), Tissue Culture Medium-199 (TCM-199) + 10% fetal calf serum, Tyrodes-Albumin-Lactate-Pyruvate (TALP), Dulbecco's Phosphate Buffered Saline (PBS), Eagle's and Whitten's media. One of the most common media used for the collection and maturation of oocytes is TCM-199, and 1 to 20% serum supplement including fetal calf serum, newborn serum, estrual cow serum, lamb serum or steer serum. A preferred maintenance medium includes TCM-199 with Earl salts, 10% fetal calf serum, 0.2 MM Ma pyruvate and 50 µg/ml gentamicin sulphate. Any of the above may also involve co-culture with a variety of cell types such as granulosa cells, oviduct cells, BRL cells and uterine cells and STO cells.

In particular, human epithelial cells of the endometrium secrete leukemia inhibitory factor (LIF) during the preimplantation and implantation period. Therefore, the addition of LIF to the culture medium could be of importance in enhancing the *in vitro* development of the reconstructed embryos. The use of LIF for embryonic or stem-like cell cultures has been described in U.S. Patent 5,712,156, which is herein incorporated by reference.

Another maintenance medium is described in U.S. Patent 5,096,822 to Rosenkrans, Jr. et al., which is incorporated herein by reference. This embryo medium, named CR1, contains the nutritional substances necessary to support an embryo. CR1 contains hemicalcium L-lactate in amounts ranging from 1.0 mM to 10 mM, preferably 1.0 mM to 5.0 mM. Hemicalcium L-lactate is L-lactate with a hemicalcium salt incorporated thereon.

Also, suitable culture medium for maintaining human embryonic cells in culture as discussed in Thomson et al., Science 282:1145-1147 (1998) and Proc. Natl. Acad. Sci., USA 92:7844-7848 (1995).

Afterward, the cultured NT unit or units are preferably washed and then placed in a suitable media, e.g., CR1aa medium, Ham's F-10, Tissue Culture Media -199 (TCM-199). Tyrodes-Albumin-Lactate-Pyrovate (TALP) Dulbecco's Phosphate Buffered Saline (PBS), Eagle's or Whitten's, preferably containing about 10% FCS. Such culturing will preferably be effected in well plates which contain a suitable confluent feeder layer. Suitable feeder layers include, by way of example, fibroblasts and epithelial cells, e.g., fibroblasts and uterine epithelial cells derived from ungulates, chicken fibroblasts, murine (e.g., mouse or rat) fibroblasts, STO and SI-m220 feeder cell lines, and BRL cells.

In the preferred embodiment, the feeder cells will comprise mouse embryonic fibroblasts. Means for preparation of a suitable fibroblast feeder layer are described in the example which follows and is well within the skill of the ordinary artisan.

The NT units are cultured on the feeder layer until the NT units reach a size suitable for obtaining cells which may be used to produce embryonic stem-like cells or cell colonies. Preferably, these NT units will be cultured until they reach a size of at least about 2 to 400 cells, more preferably about 4 to 128 cells, and most preferably at least about 50 cells. The culturing will be effected under suitable conditions, i.e., about 38.5°C and 5% CO₂, with the culture medium changed in order to optimize growth typically about every 2-5 days, preferably about every 3 days.

In the case of human cell/enucleated bovine oocyte derived NT units, sufficient cells to produce an ES cell colony, typically on the order of about 50

1 cells, will be obtained about 12 days after initiation of oocyte activation.
2 However, this may vary dependent upon the particular cell used as the nuclear
3 donor, the species of the particular oocyte, and culturing conditions. One skilled
4 in the art can readily ascertain visually when a desired sufficient number of cells
5 has been obtained based on the morphology of the cultured NT units.

6 In the case of human/human nuclear transfer embryos, it may be
7 advantageous to use culture medium known to be useful for maintaining human
8 cells in tissue culture. Examples of a culture media suitable for human embryo
9 culture include the medium reported in Jones et al, *Human Reprod.*, 13(1):169-
10 177 (1998), the P1-catalog #99242 medium, and the P-1 catalog #99292 medium,
11 both available from Irvine Scientific, Santa Ana, California, and those used by
12 Thomson et al. (1998) and (1995). (Id.).

13 As discussed above, the cells used in the present invention will preferably
14 comprise mammalian somatic cells, most preferably cells derived from an
15 actively proliferating (non-quiescent) mammalian cell culture. In an especially
16 preferred embodiment, the donor cell will be genetically modified by the
17 addition, deletion or substitution of a desired DNA sequence. For example, the
18 donor cell, e.g., a keratinocyte or fibroblast, e.g., of human, primate or bovine
19 origin, may be transfected or transformed with a DNA construct that provides for
20 the expression of a desired gene product, e.g., therapeutic polypeptide. Examples
21 thereof include lymphokines, e.g., IGF-I, IGF-II, interferons, colony stimulating
22 factors, connective tissue polypeptides such as collagens, genetic factors, clotting
23 factors, enzymes, enzyme inhibitors, etc.

24 Also, as discussed above, the donor cells may be modified prior to nuclear
25 transfer to achieve other desired effects, e.g., impaired cell lineage development,

enhanced embryonic development and/or inhibition of apoptosis. Examples of desirable modifications are discussed further below.

One aspect of the invention will involve genetic modification of the donor cell, e.g., a human cell, such that it is lineage deficient and therefore when used for nuclear transfer it will be unable to give rise to a viable offspring. This is desirable especially in the context of human nuclear transfer embryos, wherein for ethical reasons, production of a viable embryo may be an unwanted outcome. This can be effected by genetically engineering a human cell such that it is incapable of differentiating into specific cell lineages when used for nuclear transfer. In particular, cells may be genetically modified such that when used as nuclear transfer donors the resultant "embryos" do not contain or substantially lack at least one of mesoderm, endoderm or ectoderm tissue.

It is anticipated that this can be accomplished by knocking out or impairing the expression of one or more mesoderm, endoderm or ectoderm specific genes. Examples thereof include:

Mesoderm: SRF, MESP-1, HNF-4, beta-I integrin, MSD;

Endoderm: GATA-6, GATA-4;

Ectoderm: RNA helicase A, H beta 58.

The above list is intended to be exemplary and non-exhaustive of known genes which are involved in the development of mesoderm, endoderm and ectoderm. The generation of mesoderm deficient, endoderm deficient and ectoderm deficient cells and embryos has been previously reported in the literature. See, e.g., Arsenian et al, *EMBO J.*, Vol. 17(2):6289-6299 (1998); Saga Y, *Mech. Dev.*, Vol. 75(1-2):53-66 (1998); Holdener et al, *Development*, Vol. 120(5):1355-1346 (1994); Chen et al, *Genes Dev.* Vol. 8(20):2466-2477 (1994); Rohwedel et al, *Dev. Biol.*, 201(2):167-189 (1998) (mesoderm); Morrissey et al,

1 *Genes, Dev.*, Vol. 12(22):3579-3590 (1998); Soudais et al, *Development*, Vol.
2 121(11):3877-3888 (1995) (endoderm); and Lee et al, *Proc. Natl. Acad. Sci.*
3 *USA*, Vol. 95:(23):13709-13713 (1998); and Radice et al, *Development*, Vol.
4 111(3):801-811 (1991) (ectoderm).

5 In general, a desired somatic cell, e.g., a human keratinocyte, epithelial
6 cell or fibroblast, will be genetically engineered such that one or more genes
7 specific to particular cell lineages are "knocked out" and/or the expression of
8 such genes significantly impaired. This may be effected by known methods, e.g.,
9 homologous recombination. A preferred genetic system for effecting "knock-
10 out" of desired genes is disclosed by Capecchi et al, U.S. Patents 5,631,153 and
11 5,464,764, which reports positive-negative selection (PNS) vectors that enable
12 targeted modification of DNA sequences in a desired mammalian genome. Such
13 genetic modification will result in a cell that is incapable of differentiating into
14 a particular cell lineage when used as a nuclear transfer donor.

15 This genetically modified cell will be used to produce a lineage-defective
16 nuclear transfer embryo, i.e., that does not develop at least one of a functional
17 mesoderm, endoderm or ectoderm. Thereby, the resultant embryos, even if
18 implanted, e.g., into a human uterus, would not give rise to a viable offspring.
19 However, the ES cells that result from such nuclear transfer will still be useful
20 in that they will produce cells of the one or two remaining non-impaired lineage.
21 For example, an ectoderm deficient human nuclear transfer embryo will still give
22 rise to mesoderm and endoderm derived differentiated cells. An ectoderm
23 deficient cell can be produced by deletion and/or impairment of one or both of
24 RNA helicase A or H beta 58 genes.

25 These lineage deficient donor cells may also be genetically modified to
26 express another desired DNA sequence.

1 Thus, the genetically modified donor cell will give rise to a lineage-
2 deficient blastocyst which, when plated, will differentiate into at most two of the
3 embryonic germ layers.

4 Alternatively, the donor cell can be modified such that it is "mortal". This
5 can be achieved by expressing anti-sense or ribozyme telomerase genes. This
6 can be effected by known genetic methods that will provide for expression of
7 antisense DNA or ribozymes, or by gene knockout. These "mortal" cells, when
8 used for nuclear transfer, will not be capable of differentiating into viable
9 offspring.

10 Another preferred embodiment of the present invention is the production
11 of nuclear transfer embryos that grow more efficiently in tissue culture. This is
12 advantageous in that it should reduce the requisite time and necessary fusions to
13 produce ES cells and/or offspring (if the blastocysts are to be implanted into a
14 female surrogate). This is desirable also because it has been observed that
15 blastocysts and ES cells resulting from nuclear transfer may have impaired
16 development potential. While these problems may often be alleviated by
17 alteration of tissue culture conditions, an alternative solution is to enhance
18 embryonic development by enhancing expression of genes involved in
19 embryonic development.

20 For example, it has been reported that the gene products of the Ped type,
21 which are members of the MHC I family, are of significant importance to
22 embryonic development. More specifically, it has been reported in the case of
23 mouse preimplantation embryos that the Q7 and Q9 genes are responsible for the
24 "fast growth" phenotype. Therefore, it is anticipated that introduction of DNAs
25 that provide for the expression of these and related genes, or their human or other
26 mammalian counterparts into donor cells, will give rise to nuclear transfer

embryos that grow more quickly. This is particularly desirable in the context of cross-species nuclear transfer embryos which may develop less efficiently in tissue culture than nuclear transfer embryos produced by fusion of cells or nuclei of the same species.

In particular, a DNA construct containing the Q7 and/or Q9 gene will be introduced into donor somatic cells prior to nuclear transfer. For example, an expression construct can be constructed containing a strong constitutive mammalian promoter operably linked to the Q7 and/or Q9 genes, an IRES, one or more suitable selectable markers, e.g., neomycin, ADA, DHFR, and a poly-A sequence, e.g., bGH polyA sequence. Also, it may be advantageous to further enhance Q7 and Q9 gene expression by the inclusion of insulates. It is anticipated that these genes will be expressed early on in blastocyst development as these genes are highly conserved in different species, e.g., bovines, goats, porcine, dogs, cats, and humans. Also, it is anticipated that donor cells can be engineered to affect other genes that enhance embryonic development. Thus, these genetically modified donor cells should produce blastocysts and preimplantation stage embryos more efficiently.

Still another aspect of the invention involves the construction of donor cells that are resistant to apoptosis, i.e., programmed cell death. It has been reported in the literature that cell death related genes are present in preimplantation stage embryos. (Adams et al, *Science*, 281(5381):1322-1326 (1998)). Genes reported to induce apoptosis include, e.g., Bad, Bok, BH3, Bik, Hrk, BNIP3, Bim_L, Bad, Bid, and EGL-1. By contrast, genes that reportedly protect cells from programmed cell death include, by way of example, Bcl-XL, Bcl-w, Mcl-1, A1, Nr-13, BHRF-1, LMW5-HL, ORF16, Ks-Bcl-2, E1B-19K, and CED-9.

1 Thus, donor cells can be constructed wherein genes that induce apoptosis
2 are "knocked out" or wherein the expression of genes that protect the cells from
3 apoptosis is enhanced or turned on during embryonic development.

4 For example, this can be effected by introducing a DNA construct that
5 provides for regulated expression of such protective genes, e.g., Bcl-2 or related
6 genes during embryonic development. Thereby, the gene can be "turned on" by
7 culturing the embryo under specific growth conditions. Alternatively, it can be
8 linked to a constitutive promoter.

9 More specifically, a DNA construct containing a Bcl-2 gene operably
10 linked to a regulatable or constitutive promoter, e.g., PGK, SV40, CMV,
11 ubiquitin, or beta-actin, an IRES, a suitable selectable marker, and a poly-A
12 sequence can be constructed and introduced into a desired donor mammalian
13 cell, e.g., human keratinocyte or fibroblast.

14 These donor cells, when used to produce nuclear transfer embryos, should
15 be resistant to apoptosis and thereby differentiate more efficiently in tissue
16 culture. Thereby, the speed and/or number of suitable preimplantation embryos
17 produced by nuclear transfer can be increased.

18 Another means of accomplishing the same result is to impair the
19 expression of one or more genes that induce apoptosis. This will be effected by
20 knock-out or by the use of antisense or ribozymes against genes that are
21 expressed in and which induce apoptosis early on in embryonic development.
22 Examples thereof are identified above. Still alternatively, donor cells may be
23 constructed containing both modifications, i.e., impairment of apoptosis-inducing
24 genes and enhanced expression of genes that impede or prevent apoptosis. The
25 construction and selection of genes that affect apoptosis, and cell lines that
26 express such genes, is disclosed in U.S. Patent No. 5,646,008, Craig B.

1 Thompson et al inventors, and assigned to the University of Michigan. This
2 patent is incorporated by reference herein.

3 One means of enhancing efficiency is to select cells of a particular cell
4 cycle stage as the donor cell. It has been reported that this can have significant
5 effects on nuclear transfer efficiency. (Barnes et al, *Mol. Reprod. Devel.*,
6 36(1):33-41 (1993). Different methods for selecting cells of a particular cell
7 cycle stage have been reported and include serum starvation (Campbell et al,
8 *Nature*, 380:64-66 (1996); Wilmut et al, *Nature*, 385:810-813 (1997), and
9 chemical synchronization (Urbani et al, *Exp. Cell Res.*, 219(1):159-168 (1995).
10 For example, a particular cyclin DNA may be operably linked to a regulatory
11 sequence, together with a detectable marker, e.g., green fluorescent protein
12 (GFP), followed by the cyclin destruction box, and optionally insulation
13 sequences to enhance cyclin and marker protein expression. Thereby, cells of a
14 desired cell cycle can be easily visually detected and selected for use as a nuclear
15 transfer donor. An example thereof is the cyclin D1 gene in order to select for
16 cells that are in G1. However, any cyclin gene should be suitable for use in the
17 claimed invention. (See, e.g., King et al, *Mol. Biol. Cell*, Vol. 7(9):1343-1357
18 (1996)).

19 However, a less invasive or more efficient method for producing cells of
20 a desired cell cycle stage are needed. It is anticipated that this can be effected by
21 genetically modifying donor cells such that they express specific cyclins under
22 detectable conditions. Thereby, cells of a specific cell cycle can be readily
23 discerned from other cell cycles.

24 Cyclins are proteins that are expressed only during specific stages of the
25 cell cycle. They include cyclin D1, D2 and D3 in G1 phase, cyclin B1 and B2
26 in G2/M phase and cyclin E, A and H in S phase. These proteins are easily

1 translated and destroyed in the cytogolcytosol. This "transient" expression of
2 such proteins is attributable in part to the presence of a "destruction box", which
3 is a short amino acid sequence that is part of the protein that functions as a tag
4 to direct the prompt destruction of these proteins via the ugiquitin pathway.
5 (Adams et al, *Science*, 281 (5321):1322-1326 (1998)).

6 In the present invention, donor cells will be constructed that express one
7 or more of such cyclin genes under easily detectable conditions, preferably
8 visualizable, e.g., by the use of a fluorescent label. For example, a particular
9 cyclin DNA may be operably linked to a regulatory sequence, together with a
10 detectable marker, e.g., green fluorescent protein (GFP), followed by the cyclin
11 destruction box, and optionally insulation sequences to enhance cyclin and/or
12 marker protein expression. Thereby, cells of a desired cell cycle can be easily
13 visually detected and selected for use as a nuclear transfer donor. An example
14 thereof is the cyclin D1 gene which can be used to select for cells that are in G1.
15 However, any cyclin gene should be suitable for use in the claimed invention.
16 (See, e.g., King et al, *Mol. Biol. Cell*, Vol. 7(9):1343-1357 (1996)).

17 Still another aspect of the invention is a method for enhancing nuclear
18 transfer efficiency, preferably in a cross-species nuclear transfer process. While
19 the present inventors have demonstrated that nuclei or cells of one species when
20 inserted or fused with an enucleated oocyte of a different species can give rise
21 to nuclear transfer embryos that produce blastocysts, which embryos can give
22 rise to ES cell lines, the efficiency of such process is quite low. Therefore, many
23 fusions typically need to be effected to produce a blastocyst the cells of which
24 may be cultured to produce ES cells and ES cell lines.

25 Yet another means for enhancing the development of nuclear transfer
26 embryos *in vitro* is by optimizing culture conditions. One means of achieving

1 this result will be to culture NT embryos under conditions impede apoptosis.
2 With respect to this embodiment of the invention, it has been found that
3 proteases such as capsases can cause oocyte death by apoptosis similar to other
4 cell types. (See, Jurisicosva et al, *Mol. Reprod. Devel.*, 51(3):243-253 (1998).)

5 It is anticipated that blastocyst development will be enhanced by including
6 in culture media used for nuclear transfer and to maintain blastocysts or culture
7 preimplantation stage embryos one or more capsase inhibitors. Such inhibitors
8 include by way of example capsase-4 inhibitor I, capsase-3 inhibitor I, capsase-6
9 inhibitor II, capsase-9 inhibitor II, and capsase-1 inhibitor I. the amount thereof
10 will be an amount effective to inhibit apoptosis, e.g., 0.00001 to 5.0% by weight
11 of medium; more preferably 0.01% to 1.0% by weight of medium. Thus, the
12 foregoing methods may be used to increase the efficiency of nuclear transfer by
13 enhancing subsequent blastocyst and embryo development in tissue culture.

14 After NT units of the desired size are obtained, the cells are mechanically
15 removed from the zone and are then used to produce embryonic or stem-like
16 cells and cell lines. This is preferably effected by taking the clump of cells
17 which comprise the NT unit, which typically will contain at least about 50 cells,
18 washing such cells, and plating the cells onto a feeder layer, e.g., irradiated fibro-
19 blast cells. Typically, the cells used to obtain the stem-like cells or cell colonies
20 will be obtained from the inner most portion of the cultured NT unit which is
21 preferably at least 50 cells in size. However, NT units of smaller or greater cell
22 numbers as well as cells from other portions of the NT unit may also be used to
23 obtain ES-like cells and cell colonies.

24 It may be that a longer exposure of donor cell DNA to the oocyte's
25 cytosol would facilitate the dedifferentiation process. In this regard, recloning
26 could be accomplished by taking blastomeres from a reconstructed embryo and

1 fusing them with a new enucleated oocyte. Alternatively, the donor cell may be
2 fused with an enucleated oocyte and 4 to 6 hours later, without activation,
3 chromosomes may be removed and fused with a younger oocyte. Activation
4 would occur thereafter.

5 The cells are maintained in the feeder layer in a suitable growth medium,
6 e.g., alpha MEM supplemented with 10% FCS and 0.1 mM beta-
7 mercaptoethanol (Sigma) and L-glutamine. The growth medium is changed as
8 often as necessary to optimize growth, e.g., about every 2-3 days.

9 This culturing process results in the formation of embryonic or stem-like
10 cells or cell lines. In the case of human cell/bovine oocyte derived NT embryos,
11 colonies are observed by about the second day of culturing in the alpha MEM
12 medium. However, this time may vary dependent upon the particular nuclear
13 donor cell, specific oocyte and culturing conditions. One skilled in the art can
14 vary the culturing conditions as desired to optimize growth of the particular
15 embryonic or stem-like cells.

16 The embryonic or stem-like cells and cell colonies obtained will typically
17 exhibit an appearance similar to embryonic or stem-like cells of the species used
18 as the nuclear cell donor rather than the species of the donor oocyte. For
19 example, in the case of embryonic or stem-like cells obtained by the transfer of
20 a human nuclear donor cell into an enucleated bovine oocyte, the cells exhibit a
21 morphology more similar to mouse embryonic stem cells than bovine ES-like
22 cells.

23 More specifically, the individual cells of the human ES-line cell colony
24 are not well defined, and the perimeter of the colony is refractive and smooth in
25 appearance. Further, the cell colony has a longer cell doubling time, about twice

1 that of mouse ES cells. Also, unlike bovine and porcine derived ES cells, the
2 colony does not possess an epithelial-like appearance.

3 As discussed above, it has been reported by Thomson, in U.S. Patent
4 5,843,780, that primate stem cells are SSEA-1 (-), SSEA-4 (+), TRA-1-60 (+),
5 TRA-1-81 (+) and alkaline phosphatase (+). It is anticipated that human and
6 primate ES cells produced according to the present methods will exhibit similar
7 or identical marker expression.

8 Alternatively, that such cells are actual human or primate embryonic stem
9 cells will be confirmed based on their capability of giving rise to all of
10 mesoderm, ectoderm and endoderm tissues. This will be demonstrated by
11 culturing ES cells produced according to the invention under appropriate
12 conditions, e.g., as disclosed by Thomsen, U.S. Patent 5,843,780, incorporated
13 by reference in its entirety herein.

14 The resultant embryonic or stem-like cells and cell lines, preferably
15 human embryonic or stem-like cells and cell lines, have numerous therapeutic
16 and diagnostic applications. Most especially, such embryonic or stem-like cells
17 may be used for cell transplantation therapies. Human embryonic or stem-like
18 cells have application in the treatment of numerous disease conditions.

19 In this regard, it is known that mouse embryonic stem (ES) cells are
20 capable of differentiating into almost any cell type, e.g., hematopoietic stem
21 cells. Therefore, human embryonic or stem-like cells produced according to the
22 invention should possess similar differentiation capacity. The embryonic or
23 stem-like cells according to the invention will be induced to differentiate to
24 obtain the desired cell types according to known methods. For example, the
25 subject human embryonic or stem-like cells may be induced to differentiate into
26 hematopoietic stem cells, muscle cells, cardiac muscle cells, liver cells, cartilage

1 cells, epithelial cells, urinary tract cells, etc., by culturing such cells in
2 differentiation medium and under conditions which provide for cell differentia-
3 tion. Medium and methods which result in the differentiation of embryonic stem
4 cells are known in the art as are suitable culturing conditions.

5 For example, Palacios et al, *Proc. Natl. Acad. Sci., USA*, 92:7530-7537
6 (1995) teaches the production of hematopoietic stem cells from an embryonic
7 cell line by subjecting stem cells to an induction procedure comprising initially
8 culturing aggregates of such cells in a suspension culture medium lacking
9 retinoic acid followed by culturing in the same medium containing retinoic acid,
10 followed by transferral of cell aggregates to a substrate which provides for cell
11 attachment.

12 Moreover, Pedersen, *J. Reprod. Fertil. Dev.*, 6:543-552 (1994) is a review
13 article which references numerous articles disclosing methods for *in vitro*
14 differentiation of embryonic stem cells to produce various differentiated cell
15 types including hematopoietic cells, muscle, cardiac muscle, nerve cells, among
16 others.

17 Further, Bain et al, *Dev. Biol.*, 168:342-357 (1995) teaches *in vitro*
18 differentiation of embryonic stem cells to produce neural cells which possess
19 neuronal properties. These references are exemplary of reported methods for
20 obtaining differentiated cells from embryonic or stem-like cells. These
21 references and in particular the disclosures therein relating to methods for
22 differentiating embryonic stem cells are incorporated by reference in their
23 entirety herein.

24 Thus, using known methods and culture medium, one skilled in the art
25 may culture the subject embryonic or stem-like cells to obtain desired
26 differentiated cell types, e.g., neural cells, muscle cells, hematopoietic cells, etc.

1 In addition, the use of inducible Bcl-2 or Bcl-xl might be useful for
2 enhancing *in vitro* development of specific cell lineages. *In vivo*, Bcl-2 prevents
3 many, but not all, forms of apoptotic cell death that occur during lymphoid and
4 neural development. A thorough discussion of how Bcl-2 expression might be
5 used to inhibit apoptosis of relevant cell lineages following transfection of donor
6 cells is disclosed in U.S. Patent No. 5,646,008, which is herein incorporated by
7 reference.

8 The subject embryonic or stem-like cells may be used to obtain any
9 desired differentiated cell type. Therapeutic usages of such differentiated human
10 cells are unparalleled. For example, human hematopoietic stem cells may be
11 used in medical treatments requiring bone marrow transplantation. Such
12 procedures are used to treat many diseases, e.g., late stage cancers such as
13 ovarian cancer and leukemia, as well as diseases that compromise the immune
14 system, such as AIDS. Hematopoietic stem cells can be obtained, e.g., by fusing
15 adult somatic cells of a cancer or AIDS patient, e.g., epithelial cells or lympho-
16 cytes with an enucleated oocyte, e.g., bovine oocyte, obtaining embryonic or
17 stem-like cells as described above, and culturing such cells under conditions
18 which favor differentiation, until hematopoietic stem cells are obtained. Such
19 hematopoietic cells may be used in the treatment of diseases including cancer
20 and AIDS.

21 Alternatively, adult somatic cells from a patient with a neurological
22 disorder may be fused with an enucleated animal oocyte, e.g., a primate or
23 bovine oocyte, human embryonic or stem-like cells obtained therefrom, and such
24 cells cultured under differentiation conditions to produce neural cell lines.
25 Specific diseases treatable by transplantation of such human neural cells include,
26 by way of example, Parkinson's disease, Alzheimer's disease, ALS and cerebral

1 palsy, among others. In the specific case of Parkinson's disease, it has been
2 demonstrated that transplanted fetal brain neural cells make the proper connec-
3 tions with surrounding cells and produce dopamine. This can result in long-term
4 reversal of Parkinson's disease symptoms.

5 To allow for specific selection of differentiated cells, donor cells may be
6 transfected with selectable markers expressed via inducible promoters, thereby
7 permitting selection or enrichment of particular cell lineages when
8 differentiation is induced. For example, CD34-neo may be used for selection of
9 hematopoietic cells, Pw1-neo for muscle cells, Mash-1-neo for sympathetic
10 neurons, Mal-neo for human CNS neurons of the grey matter of the cerebral
11 cortex, etc.

12 The great advantage of the subject invention is that it provides an
13 essentially limitless supply of isogenic or syngenic human cells suitable for
14 transplantation. Therefore, it will obviate the significant problem associated with
15 current transplantation methods, i.e., rejection of the transplanted tissue which
16 may occur because of host-vs-graft or graft-vs-host rejection. Conventionally,
17 rejection is prevented or reduced by the administration of anti-rejection drugs
18 such as cyclosporine. However, such drugs have significant adverse side-effects,
19 e.g., immunosuppression, carcinogenic properties, as well as being very
20 expensive. The present invention should eliminate, or at least greatly reduce, the
21 need for anti-rejection drugs.

22 Other diseases and conditions treatable by isogenic cell therapy include,
23 by way of example, spinal cord injuries, multiple sclerosis, muscular dystrophy,
24 diabetes, liver diseases, i.e., hypercholesterolemia, heart diseases, cartilage
25 replacement, burns, foot ulcers, gastrointestinal diseases, vascular diseases,
26 kidney disease, urinary tract disease, and aging related diseases and conditions.

1 Also, human embryonic or stem-like cells produced according to the
2 invention may be used to produce genetically engineered or transgenic human
3 differentiated cells. Essentially, this will be effected by introducing a desired
4 gene or genes, which may be heterologous, or removing all or part of an
5 endogenous gene or genes of human embryonic or stem-like cells produced
6 according to the invention, and allowing such cells to differentiate into the de-
7 sired cell type. A preferred method for achieving such modification is by
8 homologous recombination because such technique can be used to insert, delete
9 or modify a gene or genes at a specific cite or cites in the stem-like cell genome.

10 This methodology can be used to replace defective genes, e.g., defective
11 immune system genes, cystic fibrosis genes, or to introduce genes which result
12 in the expression of therapeutically beneficial proteins such as growth factors,
13 lymphokines, cytokines, enzymes, etc. For example, the gene encoding brain
14 derived growth factor may be introduced into human embryonic or stem-like
15 cells, the cells differentiated into neural cells and the cells transplanted into a
16 Parkinson's patient to retard the loss of neural cells during such disease.

17 Previously, cell types transfected with BDNF varied from primary cells
18 to immortalized cell lines, either neural or non-neural (myoblast and fibroblast)
19 derived cells. For example, astrocytes have been transfected with BDNF gene
20 using retroviral vectors, and the cells grafted into a rat model of Parkinson's
21 disease (Yoshimoto et al., *Brain Research*, 691:25-36, (1995)).

22 This *ex vivo* therapy reduced Parkinson's-like symptoms in the rats up to
23 45% 32 days after transfer. Also, the tyrosine hydroxylase gene has been placed
24 into astrocytes with similar results (Lundberg et al., *Develop. Neurol.*, 139:39-53
25 (1996) and references cited therein).

1 However, such *ex vivo* systems have problems. In particular, retroviral
2 vectors currently used are down-regulated *in vivo* and the transgene is only
3 transiently expressed (review by Mulligan, *Science*, 260:926-932 (1993)). Also,
4 such studies used primary cells, astrocytes, which have finite life span and
5 replicate slowly. Such properties adversely affect the rate of transfection and
6 impede selection of stably transfected cells. Moreover, it is almost impossible
7 to propagate a large population of gene targeted primary cells to be used in
8 homologous recombination techniques.

9 By contrast, the difficulties associated with retroviral systems should be
10 eliminated by the use of human embryonic or stem-like cells. It has been demon-
11 strated previously by the subject assignee that cattle and pig embryonic cell lines
12 can be transfected and selected for stable integration of heterologous DNA. Such
13 methods are described in commonly assigned U.S. Serial No. 08/626,054, filed
14 April 1, 1996, incorporated by reference in its entirety. Therefore, using such
15 methods or other known methods, desired genes may be introduced into the sub-
16 ject human embryonic or stem-like cells, and the cells differentiated into desired
17 cell types, e.g., hematopoietic cells, neural cells, pancreatic cells, cartilage cells,
18 etc.

19 Genes which may be introduced into the subject embryonic or stem-like
20 cells include, by way of example, epidermal growth factor, basic fibroblast
21 growth factor, glial derived neurotrophic growth factor, insulin-like growth
22 factor (I and II), neurotrophin-3, neurotrophin-4/5, ciliary neurotrophic factor,
23 AFT-1, cytokine genes (interleukins, interferons, colony stimulating factors,
24 tumor necrosis factors (alpha and beta), etc.), genes encoding therapeutic
25 enzymes, collagen, human serum albumin, etc.

1 In addition, it is also possible to use one of the negative selection systems
2 now known in the art for eliminating therapeutic cells from a patient if necessary.
3 For example, donor cells transfected with the thymidine kinase (TK) gene will
4 lead to the production of embryonic cells containing the TK gene.
5 Differentiation of these cells will lead to the isolation of therapeutic cells of
6 interest which also express the TK gene. Such cells may be selectively
7 eliminated at any time from a patient upon gancyclovir administration. Such a
8 negative selection system is described in U.S. Patent No. 5,698,446, and is herein
9 incorporated by reference.

10 The subject embryonic or stem-like cells, preferably human cells, also
11 may be used as an *in vitro* model of differentiation, in particular for the study of
12 genes which are involved in the regulation of early development.

13 Also, differentiated cell tissues and organs using the subject embryonic
14 or stem-like cells may be used in drug studies.

15 Further, the subject embryonic or stem-like cells may be used as nuclear
16 donors for the production of other embryonic or stem-like cells and cell colonies.

17 In order to more clearly describe the subject invention, the following
18 examples are provided.

19 **EXAMPLE 1**

20 **MATERIALS AND METHODS**

21 **Donor Cells for Nuclear Transfer**

22 Epithelial cells were lightly scraped from the inside of the mouth of a
23 consenting adult with a standard glass slide. The cells were washed off the slide
24 into a petri dish containing phosphate buffered saline without Ca or Mg. The
25 cells were pipetted through a small-bore pipette to break up cell clumps into a
26 single cell suspension. The cells were then transferred into a microdrop of TL-

1 HEPES medium containing 10% fetal calf serum (FCS) under oil for nuclear
2 transfer into enucleated cattle oocytes.

3 Nuclear Transfer Procedures

4 Basic nuclear transfer procedures have been described previously.
5 Briefly, after slaughterhouse oocytes were matured *in vitro* the oocytes were
6 stripped of cumulus cells and enucleated with a beveled micropipette at ap-
7 proximately 18 hours post maturation (hpm). Enucleation was confirmed in TL-
8 HEPES medium plus bisbenzimidazole (Hoechst 33342, 3 µg/ml; Sigma). Individual
9 donor cells were then placed into the perivitelline space of the recipient oocyte.
10 The bovine oocyte cytoplasm and the donor nucleus (NT unit) are fused together
11 using electrofusion techniques. One fusion pulse consisting of 90 V for 15 µsec
12 was applied to the NT unit. This occurred at 24 hours post-initiation of
13 maturation (hpm) of the oocytes. The NT units were placed in CR1aa medium
14 until 28 hpm.

15 The procedure used to artificially activate oocytes has been described
16 elsewhere. NT unit activation was at 28 hpm. A brief description of the
17 activation procedure is as follows: NT units were exposed for four min to
18 ionomycin (5 µM; CalBiochem, La Jolla, CA) in TL-HEPES supplemented with
19 1 mg/ml BSA and then washed for five min in TL-HEPES supplemented with
20 30 mg/ml BSA. The NT units were then transferred into a microdrop of CR1aa
21 culture medium containing 0.2 mM DMAP (Sigma) and cultured at 38.5°C 5%
22 CO₂ for four to five hours. The NT units were washed and then placed in a
23 CR1aa medium plus 10% FCS and 6 mg/ml BSA in four well plates containing
24 a confluent feeder layer of mouse embryonic fibroblasts (described below). The
25 NT units were cultured for three more days at 38.5°C and 5% CO₂. The culture
26 medium was changed every three days until day 12 after the time of activation.

At this time NT units reaching the desired cell number, i.e., about 50 cell number, were mechanically removed from the zona and used to produce embryonic cell lines. A photograph of an NT unit obtained as described above is contained in Figure 1.

Fibroblast feeder layer

Primary cultures of embryonic fibroblasts were obtained from 14-16 day old murine fetuses. After the head, liver, heart and alimentary tract were aseptically removed, the embryos were minced and incubated for 30 minutes at 37°C in prewarmed trypsin EDTA solution (0.05% trypsin/0.02% EDTA; GIBCO, Grand Island, NY). Fibroblast cells were plated in tissue culture flasks and cultured in alpha-MEM medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum (FCS) (Hyclone, Logen, UT), penicillin (100 IU/ml) and streptomycin (50 µl/ml). Three to four days after passage, embryonic fibroblasts, in 35 x 10 Nunc culture dishes (Baxter Scientific, McGaw Park, IL), were irradiated. The irradiated fibroblasts were grown and maintained in a humidified atmosphere with 5% CO₂ in air at 37°C. The culture plates which had a uniform monolayer of cells were then used to culture embryonic cell lines.

Production of embryonic cell line.

NT unit cells obtained as described above were washed and plated directly onto irradiated feeder fibroblast cells. These cells included those of the inner portion of the NT unit. The cells were maintained in a growth medium consisting of alpha MEM supplemented with 10% FCS and 0.1 mM beta-mercaptoethanol (Sigma). Growth medium was exchanged every two to three days. The initial colony was observed by the second day of culture. The colony was propagated and exhibits a similar morphology to previously disclosed mouse embryonic stem (ES) cells. Individual cells within the colony are not well

defined and the perimeter of the colony is refractile and smooth in appearance. The cell colony appears to have a slower cell doubling time than mouse ES cells. Also, unlike bovine and porcine derived ES cells, the colony does not have an epithelial appearance thus far. Figures 2 through 5 are photographs of ES-like cell colonies obtained as described, *supra*.

Production of Differentiated Human Cells

The human embryonic cells obtained are transferred to a differentiation medium and cultured until differentiated human cell types are obtained.

RESULTS

Table 1. Human cells as donor nuclei in NT unit production and development.

TABLE 1

Cell type	No. NT units made	No. NT units 2 cell stage (%)	No. NT units to 4 - 16 cell stage (%)	No. NT units to 16 - 400 cell stage (%)
lymphocytes	18	12 (67%)	3 (17%)	0
oral cavity epithelium	34	18 (53%)	3 (9%)	1 (3%)
adult fibroblasts	46	4 (9%)	12 (4 cell; 26%) 8 (8-16 cells; 17.4%)	---

The one NT unit that developed a structure having greater than 16 cells was plated down onto a fibroblast feeder layer. This structure was attached to the feeder layer and started to propagate forming a colony with a ES cell-like morphology (See, e.g., Figure 2). Moreover, although the 4 to 16 cell stage structures were not used to try and produce an ES cell colony, it has been previously shown that this stage is capable of producing ES or ES-like cell lines (mouse, Eistetter et al., *Devel. Growth and Differ.*, 31:275-282 (1989); Bovine,

1 Stice et al., 1996)). Therefore, it is expected that 4 - 16 cell stage NT units
2 should also give rise to embryonic or stem-like cells and cell colonies.

3 Also, similar results were obtained upon fusion of an adult human
4 keratinocyte cell line with an enucleated bovine oocyte, which was cultured in
5 media comprising ACM, uridine, glucose, and 1000 IU of LIF. Out of 50
6 reconstructed embryos, 22 cleaved and one developed into a blastocyst at about
7 day 12. This blastocyst was plated and the production of an ES cell line is
8 ongoing.

9 While the present invention has been described and illustrated herein by
10 reference to various specific materials, procedures, and examples, it is under-
11 stood that the invention is not restricted to the particular material, combinations
12 of materials, and procedures selected for that purpose. Numerous variations of
such details can be implied and will be appreciated by those skilled in the art.

WHAT IS CLAIMED IS:

1. A method of producing embryonic or stem-like cells comprising the following steps:
 - (i) inserting a desired differentiated human or mammalian cell or cell nucleus into an enucleated animal oocyte, wherein such oocyte is derived from a different animal species than the human or mammalian cell under conditions suitable for the formation of a nuclear transfer (NT) unit;
 - (ii) activating the resultant nuclear transfer unit;
 - (iii) culturing said activated nuclear transfer unit until greater than the 2-cell developmental stage; and
 - (iv) culturing cells obtained from said cultured NT units to obtain embryonic or stem-like cells.
2. The method of Claim 1, wherein the cell inserted into the enucleated animal oocyte is a human cell.
3. The method of Claim 2, wherein said human cell is an adult cell.
4. The method of Claim 2, wherein said human cell is an epithelial cell, keratinocyte, lymphocyte or fibroblast.
5. The method of Claim 2, wherein the oocytes are obtained from a mammal.

15. The method of Claim 1, wherein the resultant embryonic or stem-like cells are induced to differentiate.

16. The method of Claim 2, wherein the resultant embryonic or stem-like cells are induced to differentiate.

17. The method of Claim 1, wherein fusion is effected by electrofusion.

18. Embryonic or stem-like cells obtained according to the method of Claim 1.

19. Human embryonic or stem-like cells obtained according to the method of Claim 2.

20. Human embryonic or stem-like cells obtained according to the method of Claim 3.

21. Human embryonic or stem-like cells obtained according to the method of Claim 4.

22. Human embryonic or stem-like cells obtained according to the method of Claim 6.

23. Human embryonic or stem-like cells obtained according to the method of Claim 7.

24. Differentiated human cells obtained by the method of Claim 16.

25. The differentiated human cells of Claim 24, which are selected from the group consisting of neural cells, hematopoietic cells, pancreatic cells, muscle cells, cartilage cells, urinary cells, liver cells, spleen cells, reproductive cells, skin cells, intestinal cells, and stomach cells.

26. A method of therapy which comprises administering to a patient in need of cell transplantation therapy isogenic differentiated human cells according to Claim 24.

27. The method of Claim 26, wherein said cell transplantation therapy is effected to treat a disease or condition selected from the group consisting of Parkinson's disease, Huntington's disease, Alzheimer's disease, ALS, spinal cord defects or injuries, multiple sclerosis, muscular dystrophy, cystic fibrosis, liver disease, diabetes, heart disease, cartilage defects or injuries, burns, foot ulcers, vascular disease, urinary tract disease, AIDS and cancer.

28. The method of Claim 26, wherein the differentiated human cells are hematopoietic cells or neural cells.

29. The method of Claim 26, wherein the therapy is for treatment of Parkinson's disease and the differentiated cells are neural cells.

30. The method of Claim 26, wherein the therapy is for the treatment of cancer and the differentiated cells are hematopoietic cells.

31. The differentiated human cells of Claim 24, which contain and express an inserted gene.

32. The method of Claim 1, wherein a desired gene is inserted, removed or modified in said embryonic or stem-like cells.

33. The method of Claim 32, wherein the desired gene encodes a therapeutic enzyme, a growth factor or a cytokine.

34. The method of Claim 32, wherein said embryonic or stem-like cells are human embryonic or stem-like cells.

35. The method of Claim 32, wherein the desired gene is removed, modified or deleted by homologous recombination.

36. The method of Claim 1, wherein the donor cell is genetically modified to impair the development of at least one of endoderm, ectoderm and mesoderm.

37. The method of Claim 1, wherein the donor cell is genetically modified to increase differentiation efficiency.

38. The method of Claim 36, wherein the cultured nuclear transfer unit is cultured in a media containing at least one capsase inhibitor.

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39. The method of Claim 1, wherein the donor cell expresses a detectable label that is indicative of the expression of a particular cyclin.

40. The method of Claim 36, wherein the donor cell has been modified to alter the expression of a gene selected from the group consisting of SRF, MESP-1, HNF-4, beta-1, integrin, MSD, GATA-6, GATA-4, RNA helicase A, and H beta 58.

41. The method of Claim 37, wherein said donor cell has been genetically modified to introduce a DNA that provides for expression of the Q7 and/or Q9 genes.

42. The method of Claim 41, wherein said gene or genes are operably linked to a regulatable promoter.

43. The method of Claim 1, wherein the donor cell has been genetically modified to inhibit apoptosis.

44. The method of Claim 43, wherein reduced apoptosis is provided by altering expression of one or more genes selected from the group consisting of Bad, Bok, BH3, Bik, Blk, Hrk, BNIP3, Gim_L, Bid, EGL-1, Bcl-XL, Bcl-w, Mcl-1, A1, Nr-13, BHRF-1, LMW5-HL, ORF16, Ks-Bcl-2, E1B-19K, and CED-9.

45. The method of Claim 44, wherein at least one of said genes is operably linked to an inducible promoter.

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46. A mammalian somatic cell that expresses a DNA that encodes a detectable marker, the expression of which is linked to a particular cyclin.

47. The cell of Claim 46, wherein the cyclin is selected from the group consisting of cyclin D1, D2, D3, B1, B2, E, A and H.

48. The cell of Claim 46, wherein the detectable marker is a fluorescent polypeptide.

49. The cell of Claim 48, wherein said mammalian cell is selected from the group consisting of human, primate, rodent, ungulate, canine, and feline cells.

50. The cell of Claim 48, wherein said cell is a human, bovine or primate cell.

An improved method of nuclear transfer involving the transplantation of differentiated donor cell nuclei into enucleated oocytes of a species different from the donor cell is provided. The resultant nuclear transfer units are useful for the production of isogenic embryonic stem cells, in particular human isogenic embryonic or stem cells. These embryonic or stem-like cells are useful for producing desired differentiated cells and for introduction, removal or modification, of desired genes, e.g., at specific sites of the genome of such cells by homologous recombination. These cells, which may contain a heterologous gene, are especially useful in cell transplantation therapies and for *in vitro* study of cell differentiation. Also, methods for improving nuclear transfer efficiency by genetically altering donor cells to inhibit apoptosis, select for a specific cell cycle and/or enhance embryonic growth and development are provided.

FIGURE 1

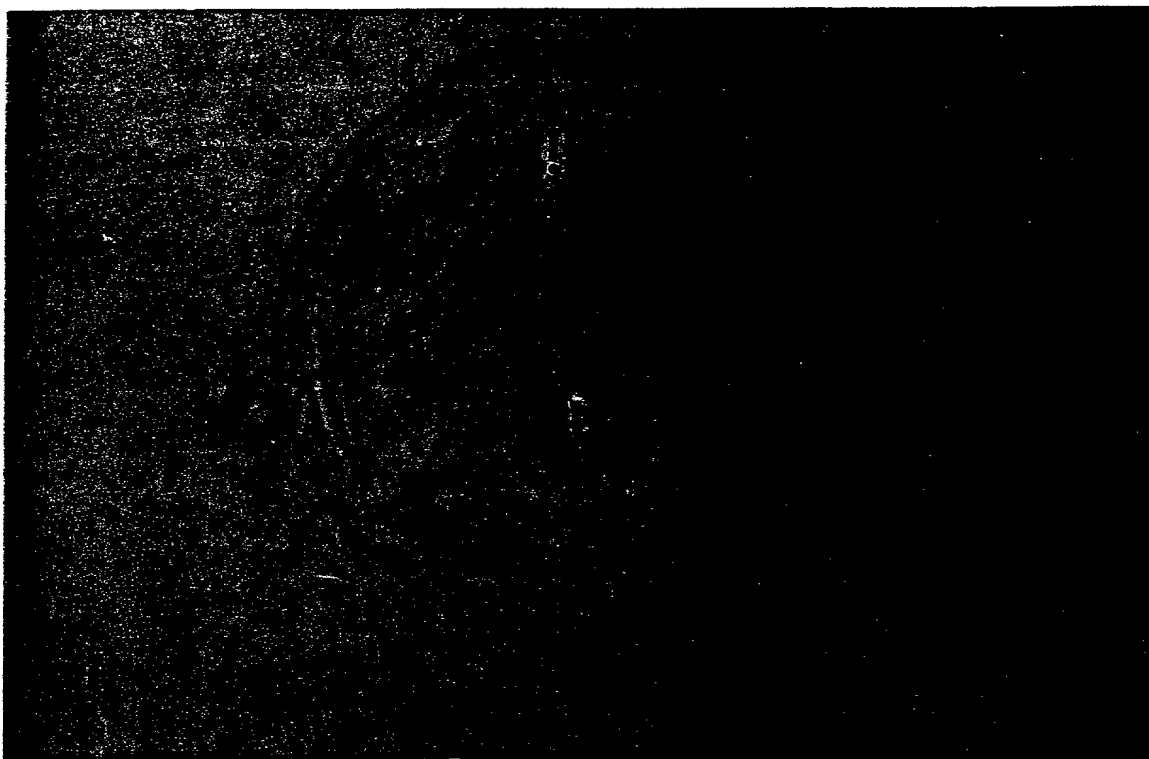
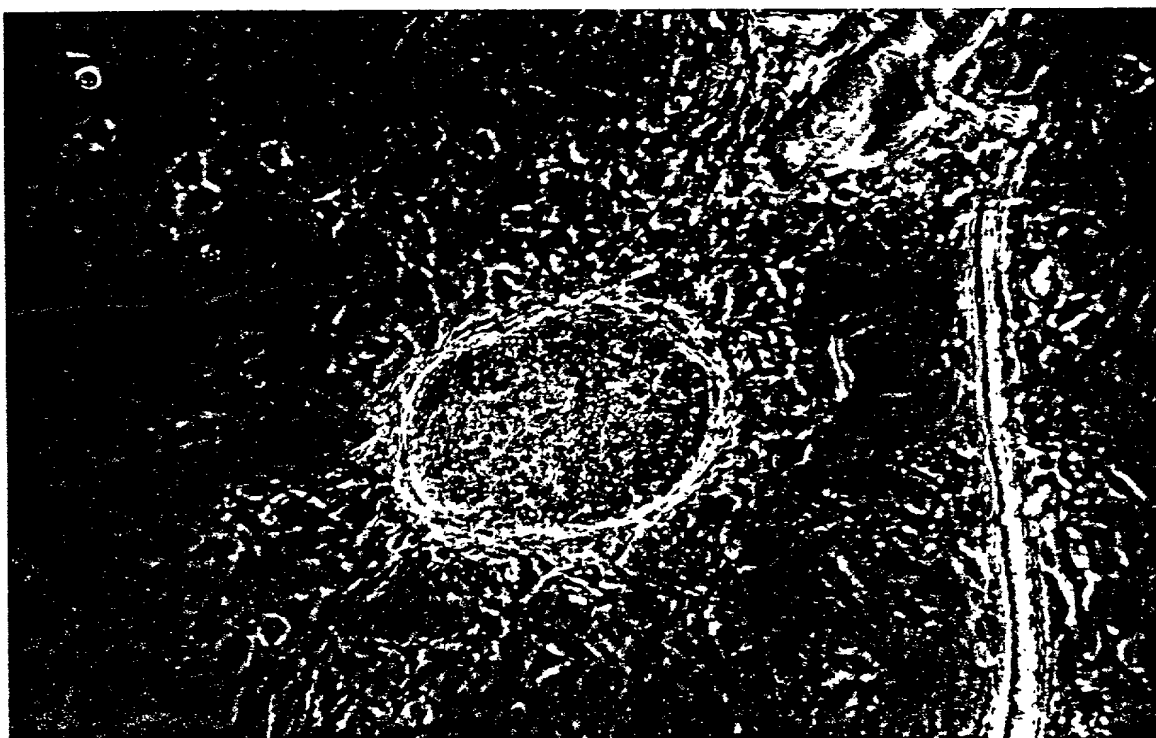


FIGURE 2



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FIGURE 3

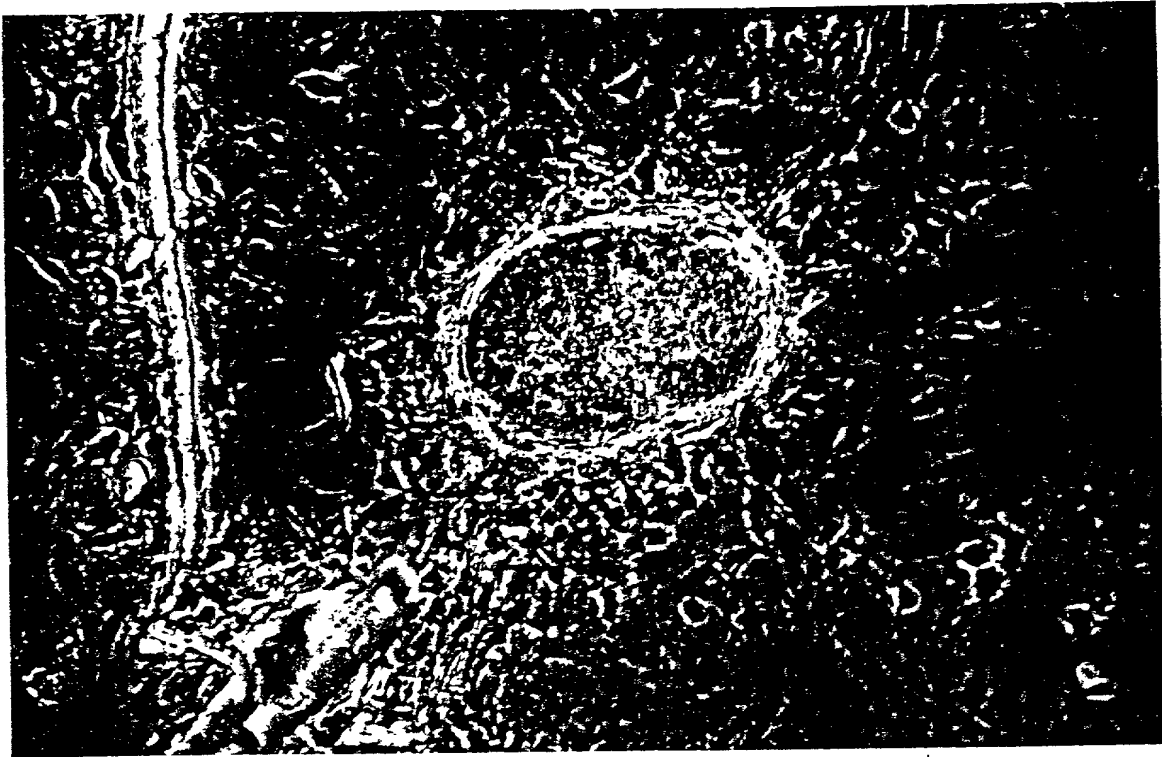
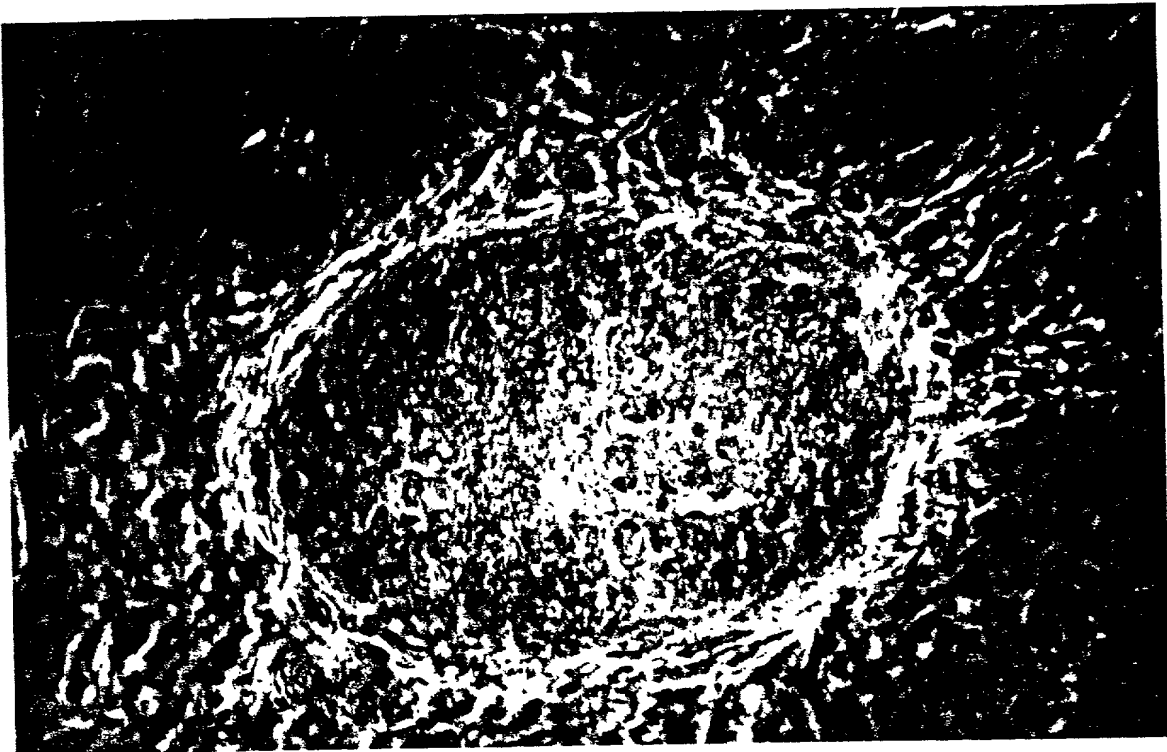
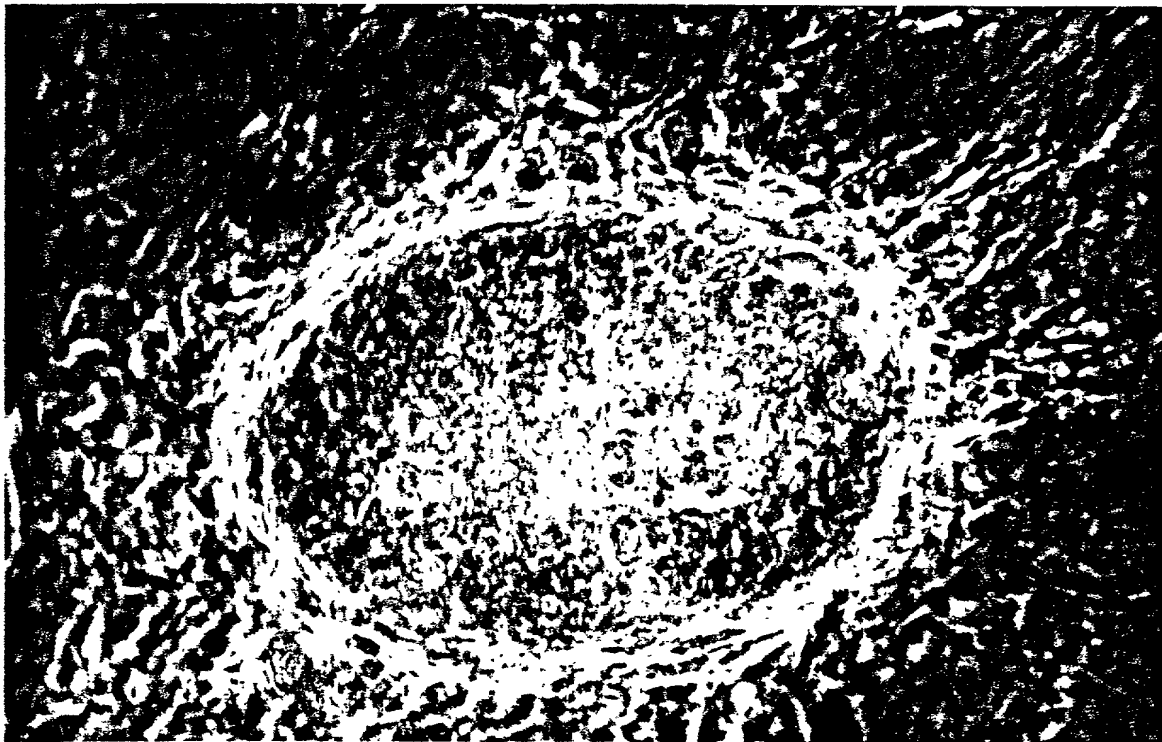


FIGURE 4



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FIGURE 5



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As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

EMBRYONIC OR STEM-LIKE CELL LINES PRODUCED BY CROSS-SPECIES NUCLEAR TRANSPLANTATION

AND METHODS FOR ENHANCING EMBRYONIC DEVELOPMENT BY GENETIC ALTERATION OF DONOR CELLS
OR BY TISSUE CULTURE CONDITIONS

the specification of which (check only one item below):

☒ is attached hereto.

☐ was filed as United States application

Number _____

on _____

and was amended

on _____ (if applicable).

☐ was filed as PCT international application

Number _____

on _____

and was amended under PCT Article 19

on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(e) of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. § 119:

COUNTRY (if PCT, indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 U.S.C. § 119
			__ Yes __ No
			__ Yes __ No
			__ Yes __ No
			__ Yes __ No
			__ Yes __ No

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below.

(Application Number)

(Filing Date)

(Application Number)

(Filing Date)

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (CONTINUED) (Includes Reference to Provisional and PCT International Applications)				ATTORNEY'S DOCKET NO. 000270-057	
I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose to the Office all information known to me to be material to the patentability as defined in Title 37, Code of Federal Regulations §1.56, which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application:					
PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:					
U.S. APPLICATIONS			STATUS <i>(check one)</i>		
U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	PENDING	ABANDONED	
09/030,945	March 2, 1998		X		
08/699,040	August 19, 1996		X		
PCT APPLICATIONS DESIGNATING THE U.S.					
PCT APPLICATION NO.	PCT FILING DATE	U.S. APPLICATION NUMBERS ASSIGNED (if any)			
I hereby appoint the following attorneys and agent(s) to prosecute said application and to transact all business in the Patent and Trademark Office connected therewith and to file, prosecute and to transact all business in connection with international applications directed to said invention:					
William L. Mathis Peter H. Smolka Robert S. Swecker Platon N. Mandros Benton S. Duffett, Jr. Norman H. Stepno Ronald L. Grudziecki Frederick G. Michaud, Jr. Alan E. Kopecki Regis E. Slutter Samuel C. Miller, III Ralph L. Freeland, Jr. Robert G. Mukai	17,337 15,913 19,885 22,124 22,030 22,716 24,970 26,003 25,813 26,999 27,360 16,110 28,531	George A. Hovanec, Jr. James A. LaBarre E. Joseph Gess R. Danny Huntington Eric H. Weisblatt James W. Peterson Teresa Stanek Rea Robert E. Krebs William C. Rowland T. Gene Dillahunt Patrick C. Keane Bruce J. Boggs, Jr. William H. Benz	28,223 28,632 28,510 27,903 30,505 26,057 30,427 25,885 30,888 25,423 32,858 32,344 25,952	Peter K. Skiff Richard J. McGrath Matthew L. Schneider Michael G. Savage Gerald F. Swiss Michael J. Ure Charles F. Wieland III Bruce T. Wieder Todd R. Walters Ronni S. Jillions Harold R. Brown III Allen R. Baum Steven M. du Bois	31,917 29,195 32,814 32,596 30,113 33,089 33,096 33,815 34,040 31,979 36,341 36,086 35,023
and: <u>Robin L. Teskin, Registration No. 35,030</u>					
Address all correspondence to:		Robin L. Teskin BURNS, DOANE, SWECKER & MATHIS, L.L.P. P.O. Box 1404 Alexandria, Virginia 22313-1404			
Address all telephone calls to:		<u>Robin L. Teskin</u> at (703) 836-6620.			
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.					

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (CONTINUED) (Includes Reference to Provisional and PCT International Applications)		ATTORNEY'S DOCKET NO. 000270-057
FULL NAME OF SOLE OR FIRST INVENTOR James ROBL	SIGNATURE	DATE
RESIDENCE Belchertown, Massachusetts	CITIZENSHIP U.S.A.	
POST OFFICE ADDRESS 196 Old Enfield, Belchertown, MA 01002		
FULL NAME OF SECOND JOINT INVENTOR, IF ANY Jose CIBELL	SIGNATURE	DATE
RESIDENCE Amherst, Massachusetts	CITIZENSHIP Argentinian	
POST OFFICE ADDRESS 166 Village Park, Amherst, MA 01002		
FULL NAME OF THIRD JOINT INVENTOR, IF ANY Steven L. STICE	SIGNATURE	DATE
RESIDENCE Belchertown, Massachusetts	CITIZENSHIP U.S.A.	
POST OFFICE ADDRESS 468 Amherst Road, Belchertown, MA 01007		
FULL NAME OF FOURTH JOINT INVENTOR, IF ANY	SIGNATURE	DATE
RESIDENCE	CITIZENSHIP	
POST OFFICE ADDRESS		
FULL NAME OF FIFTH JOINT INVENTOR, IF ANY	SIGNATURE	DATE
RESIDENCE	CITIZENSHIP	
POST OFFICE ADDRESS		
FULL NAME OF SIXTH JOINT INVENTOR, IF ANY	SIGNATURE	DATE
RESIDENCE	CITIZENSHIP	
POST OFFICE ADDRESS		
FULL NAME OF SEVENTH JOINT INVENTOR, IF ANY	SIGNATURE	DATE
RESIDENCE	CITIZENSHIP	
POST OFFICE ADDRESS		
FULL NAME OF EIGHTH JOINT INVENTOR, IF ANY	SIGNATURE	DATE
RESIDENCE	CITIZENSHIP	
POST OFFICE ADDRESS		
FULL NAME OF NINTH JOINT INVENTOR, IF ANY	SIGNATURE	DATE
RESIDENCE	CITIZENSHIP	
POST OFFICE ADDRESS		